GNE.3230R1C10   PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Goddard, et al. (as amended)
Appl. No.	:	10/063,518
Filed	:	May 1, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	Larry Ronald Helms
Group Art Unit	:	1642

DECLARATION UNDER 37 C.F.R. §1.808

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I hereby aver that the nucleic acid of SEQ ID NO: 13 was deposited with the American Type Culture Collection (ATCC®) January 12, 1999 and was given ATCC® deposit number 203579. Accordingly, the deposited material has been accepted for deposit under the Budapest Treaty on the International Recognition of the deposit of Microorganisms for the Purposes of Patent Procedure and all restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the grant of a patent from this application.

GENENTECH, INC.

Date: _4/26/05_   By: _____

                                   Title: _PATENT AGENT_

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Exhibit 1

GNE.3230R1C39

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Eaton, et al.
Appl. No.	:	10/063,557
Filed	:	May 2, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	David J. Blanchard
Group Art Unit	:	1642

DECLARATION OF J. CHRISTOPHER GRIMALDI, UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, J. Christopher Grimaldi, declare and state as follows:

1.   I am a Senior Research Associate in the Molecular Biology Department of Genentech, Inc., South San Francisco, CA 94080.

2.   My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).

3.   I joined Genentech in January of 1999. From 1999 to 2003, I directed the Cloning Laboratory in the Molecular Biology Department. During this time I directed or performed numerous molecular biology techniques including semi-quantitative Polymerase Chain Reaction (PCR) analyses. I am currently involved, among other projects, in the isolation of genes coding for membrane associated proteins which can be used as targets for antibody therapeutics against cancer. In connection with the above-identified patent application, I personally performed or directed the semi-quantitative PCR gene expression analyses in the assay entitled "Tumor Versus Normal Differential Tissue Expression Distribution," which is described in EXAMPLE 18 in the specification. These studies were used to identify differences in gene expression between tumor tissue and their normal counterparts.

4.   EXAMPLE 18 reports the results of the PCR analyses conducted as part of the investigating of several newly discovered DNA sequences. This process included developing

primers and analyzing expression of the DNA sequences of interest in normal and tumor tissues. The analyses were designed to determine whether a difference exists between gene expression in normal tissues as compared to tumor in the same tissue type.

5.   The DNA libraries used in the gene expression studies were made from pooled samples of normal and of tumor tissues. Data from pooled samples is more likely to be accurate than data obtained from a sample from a single individual. That is, the detection of variations in gene expression is likely to represent a more generally relevant condition when pooled samples from normal tissues are compared with pooled samples from tumors in the same tissue type.

6.   In differential gene expression studies, one looks for genes whose expression levels differ significantly under different conditions, for example, in normal versus diseased tissue. Thus, I conducted a semi-quantitative analysis of the expression of the DNA sequences of interest in normal versus tumor tissues. Expression levels were graded according to a scale of +, - , and +/- to indicate the amount of the specific signal detected. Using the widely accepted technique of PCR, it was determined whether the polynucleotides tested were more highly expressed, less expressed, or whether expression remained the same in tumor tissue as compared to its normal counterpart. Because this technique relies on the visual detection of ethidium bromide staining of PCR products on agarose gels, it is reasonable to assume that any detectable differences seen between two samples will represent at least a two fold difference in cDNA.

7.   The results of the gene expression studies indicate that the genes of interest can be used to differentiate tumor from normal. The precise levels of gene expression are irrelevant; what matters is that there is a relative difference in expression between normal tissue and tumor tissue. The precise type of tumor is also irrelevant; again, the assay was designed to indicate whether a difference exists between normal tissue and tumor tissue of the same type. If a difference is detected, this indicates that the gene and its corresponding polypeptide and antibodies against the polypeptide are useful for diagnostic purposes, to screen samples to differentiate between normal and tumor. Additional studies can then be conducted if further information is desired.

8.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____   Date:_____

J. Christopher Grimaldi

S:\DOCS\AOK\AOK\5591.DOC/081004

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J. Christopher Grimaldi

1434-36th Ave.
San Francisco, CA 94122
(415) 681-1639 (Home)

EDUCATION University of California, Berkeley
Bachelor of Arts in Molecular Biology, 1984

EMPLOYMENT EXPERIENCE

SRA Genentech Inc., South San Francisco; 1/99 to present

Previously, was responsible to direct and manage the Cloning Lab. Currently focused on isolating cancer specific genes for the Tumor Antigen (TAP), and Secreted Tumor Protein (STOP) projects for the Oncology Department as well as Immunologically relevant genes for the Immunology Department. Directed a lab of 6 scientists focused on a company-wide team effort to identify and isolate secreted proteins for potential therapeutic use (SPDI). For the SPDI project my duties were, among other things, the critically important coordination of the cloning of thousands of putative genes, by developing a smooth process of communication between the Bioinformatics, Cloning, Sequencing, and Legal teams. Collaborated with several groups to discover novel genes through the Curagen project, a unique differential display methodology. Interacted extensively with the Legal team providing essential data needed for filing patents on novel genes discovered through the SPDI, TAP and Curagen projects. My group has developed, implemented and patented high throughput cloning methodologies that have proven to be essential for the isolation of hundreds of novel genes for the SPDI, TAP and Curagen projects as well as dozens of other smaller projects.

Scientist DNAX Research Institute, Palo Alto; 9/91 to 1/99

Involved in multiple projects aimed at understanding novel genes discovered through bioinformatics studies and functional assays. Developed and patented a method for the specific depletion of eosinophils in vivo using monoclonal antibodies. Developed and implemented essential technical methodologies and provided strategic direction in the areas of expression, cloning, protein purification, general molecular biology, and monoclonal antibody production. Trained and supervised numerous technical staff.

Facilities Manager Corixa, Redwood City; 5/89 - 7/91.

Directed plant-related activities, which included expansion planning, maintenance, safety, purchasing, inventory control, shipping and receiving, and laboratory management. Designed and implemented the safety program. Also served as liaison to regulatory agencies at the local, state and federal level. Was in charge of property leases, leasehold improvements, etc. Negotiated vendor contracts and directed the purchasing department. Trained and supervised personnel to carry out the above-mentioned duties.

SRA   University of California, San Francisco
Cancer Research Institute; 2/87-4/89.

Was responsible for numerous cloning projects including: studies of somatic hypermutation, studies of AIDS-associated lymphomas, and cloning of t(5;14), t(11;14), and t(8;14) translocations. Focused on the activation of hemopoietic growth factors involved in the t(5;14) translocation in leukemia patients..

Research
Technician   Berlex Biosciences, South San Francisco; 7/85-2/87.

Worked on a subunit porcine vaccine directed against Mycoplasma hyopneumoniae. Was responsible for generating genomic libraries, screening with degenerate oligonucleotides, and characterizing and expressing clones in E. coli. Also constructed a general purpose expression vector for use by other scientific teams.

PUBLICATIONS

1. Hilary F. Clark, et al. "The Secreted Protein Discovery Initiative (SPDI), a Large-scale Effort to Identify Novel Human Secreted and Transmembrane Proteins: a bioinformatics assessment." Genome Res. Vol 13(10), 2265-2270, 2003

2. Sean H. Adams, Clarissa Chui Sarah L. Schilbach, Xing Xian Yu, Audrey D. Goddard, J. Christopher Grimaldi, James Lee, Patrick Dowd, David A. Lewin, & Steven Colman "BFIT, a Unique Acyl-CoA Thioesterase Induced in Thermogenic Brown Adipose Tissue: Cloning, organization of the humanb gene and assessment of a potential link to obesity" Biochemical Journal, Vol 360, 135-142, 2001

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4. Jeanne Kahn, Fuad Mehraban, Gladdys Ingle, Xiaohua Xin, Juliet E. Bryant, Gordon Vehar, Jill Schoenfeld, J. Christopher Grimaldi (incorrectly named as "Grimaldi, CJ"), Franklin Peale, Aparna Draksharapu, David A. Lewin, and Mary E. Gerritsen. "Gene Expression Profiling in an in Vitro Model of Angiogenesis." American Journal of Pathology Vol 156(6), 1887-1900, 2000.

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9. M. J. Guimaraes, J. F. Bazan, A. Zolotnik, M. V. Wiles, J. C. Grimaldi, F. Lee, T. McClanahan. "A new approach to the study of haematopoietic development in the yolk sac and embryoid body." Development, Vol. 121(10), 3335-3346, 1995

10. J. Christopher Grimaldi, Sriram Balasubramanian, J. Fernando Bazan, Armen Shanafelt, Gerard Zurawski and Maureen Howard. "CD38-mediated protein ribosylation." Journal of Immunology, Vol. 155(2), 811-817, 1995

11. Leopoldo Santos-Argumedo, Frances F. Lund, Andrew W. Heath, Nanette Solvason, Wei Wei Wu, J. Christopher Grimaldi, R. M. E. Parkhouse and Maureen Howard. "CD38 unresponsiveness of xid B cells implicates Bruton's tyrosine kinase (btk) as a regulator of CD38 induced signal transduction." International Immunology, Vol 7(2), 163-170, 1995

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14. Nobuyuki Harada, Leopoldo Santos-Argumedo, Ray Chang, J. Christopher Grimaldi, Frances Lund, Camilynn I. Brannan, Neal G. Copeland, Nancy A. Jenkins, Andrew Heath, R. M. E. Parkhouse and Maureen Howard. "Expression Cloning of a cDNA Encoding a Novel Murine B Cell Activation Marker: Homology to Human CD38." The Journal of Immunology, Vol. 151, 3111-3118, 1993

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Division into Peripheral Monoclonal, Polyclonal and Central Nervous System Lymphoma." AIDS, Vol. 5, 669-674, 1991

18. Ann Grimaldi and Chris Grimaldi. "Small-Scale Lambda DNA Prep." Contribution to Current Protocols in Molecular Biology, Supplement 5, Winter 1989

19. J. Christopher Grimaldi, Timothy C. Meeker. "The t(5;14) Chromosomal Translocation in a Case of Acute Lymphocytic Leukemia Joins the Interleukin-3 Gene to the Immunoglobulin Heavy Chain Gene." Blood, Vol. 73, 2081-2085, 1989

20. Timothy C. Meeker, J. Christopher Grimaldi, et al. "An Additional Breakpoint Region in the BCL-1 Locus Associated with the t(11;14) (q13;q32) Translocation of B-Lymphocytic Malignancy." Blood, Vol. 74, 1801-1806, 1989

21 Timothy C. Meeker, J. Christopher Grimaldi, Robert O'Rourke, et al. "Lack of Detectable Somatic Hypermutation in the V Region of the Ig H Chain Gene of a Human Chronic B Lymphocytic Leukemia." The Journal of Immunology, Vol. 141, 3994-3998, 1988

MANUSCRIPTS IN PREPARATION

1. Sriram Balasubramanian, J. Christopher Grimaldi, J. Fernando Bazan, Gerard Zurawski and Maureen Howard. "Structural and functional characterization of CD38: Identification of active site residues"

PATENTS

1. "Methods for Eosinophil Depletion with Antibody to CCR3 Receptor" (US 6,207,155 B1).

2. "Amplification Based Cloning Method." (US 6,607,899)

3. Ashkenazi et al., "Secreted and Transmembrane Polypeptides and Nucleic Acids Encoding the Same." (this patent covers several hundred genes)

4. "IL-17 Homologous Polypeptides and Therapeutic Uses Thereof"

5. "Method of Diagnosing and Treating Cartilaginous Disorders."

MEMBERSHIPS AND ACTIVITIES

Editor   Frontiers in Bioscience

Member   DNAX Safety Committee 1991-1999
Biological Safety Affairs Forum (BSAF) 1990-1991
Environmental Law Foundation (ELF) 1990-1991

The t(5;14) Chromosomal Translocation in a Case of Acute Lymphocytic Leukemia Joins the Interleukin-3 Gene to the Immunoglobulin Heavy Chain Gene

By J. Christopher Grimaldi and Timothy C. Meeker

Chromosomal translocations have proven to be important markers of the genetic abnormalities central to the pathogenesis of cancer. By cloning chromosomal breakpoints one can identify activated proto-oncogenes. We have studied a case of B-lineage acute lymphocytic leukemia (ALL) that was associated with peripheral blood eosinophilia. The chromosomal translocation t(5;14) (q31;q32) from this sample was cloned and studied at the molecular level. This translocation joined the immunoglobulin heavy chain joining (Jh) region to the promotor region of the interleukin-3 (IL-3) gene in opposite transcriptional orientations. The data suggest that activation of the IL-3 gene by the enhancer of the immunoglobulin heavy chain gene may play a central role in the pathogenesis of this leukemia and the associated eosinophilia.

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KARYOTYPIC STUDIES of leukemia and lymphoma have identified frequent nonrandom chromosomal translocations. Some of these translocations juxtapose the immunoglobulin heavy chain (IgH) gene with important
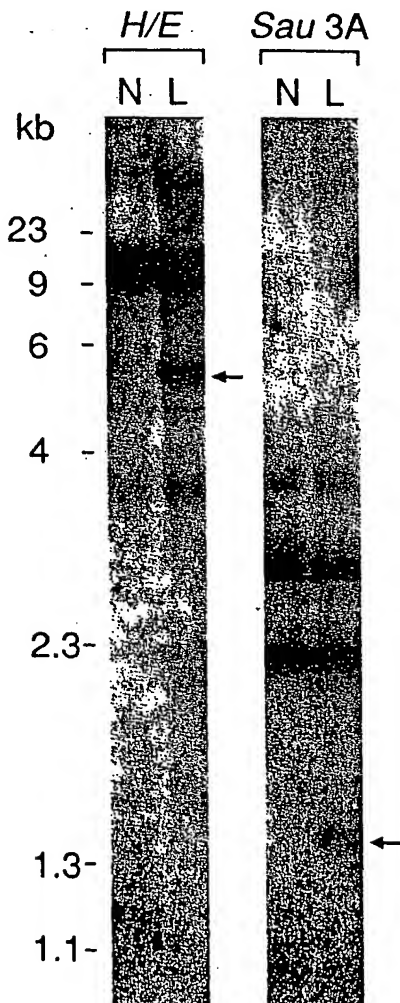

Fig 1. DNA blots of the leukemia sample. The restriction fragment pattern of normal human DNA (N) and the leukemia sample (L) were compared using a human Jh probe. Rearranged bands are indicated by arrows. Sample L exhibits a single rearranged band with both Hind III/EcoRI and Sau3A restriction digests. The rearranged bands are less intense than the other bands because the majority of cells in the sample represent normal bone marrow elements.

protooncogenes, such as c-*myc* and *bcl*-2.[1,2] In this way, the IgH gene can activate proto-oncogenes, resulting in disordered gene expression and a step in the development of cancer. The investigation of additional nonrandom translocations into the IgH locus allows us to identify new genes promoting the generation of leukemia and lymphoma.

A distinct subtype of acute lymphocytic leukemia (ALL) has been characterized by B-lineage phenotype, associated eosinophilia in the peripheral blood, and a t(5;14)(q31;q32) chromosomal translocation.[3,4] This syndrome probably occurs in <1% of all patients with ALL. We hypothesized that the cloning of the translocation characteristic of this leukemia might allow the identification of an important gene on chromosome 5 that plays a role in the evolution of this disease. In this report we demonstrate that the interleukin-3 gene (IL-3) and the IgH gene are joined by this translocation.

MATERIALS AND METHODS

Sample and DNA blots. A bone marrow aspirate from a representative patient with ALL (L1 morphology by French-American-British [FAB] criteria), peripheral eosinophilia (up to 20,000 per microliter with a normal value of <350 per microliter) and a t(5;14)(q31;q32) translocation was studied. Using published methods, genomic DNA was isolated and DNA blots were made.[5] Briefly, 10 μg of high molecular weight (mol wt) DNA were digested using an appropriate restriction enzyme and electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide, photographed, denatured, neutralized, and transferred to Hybond (Amersham, Arlington Heights, IL). After treatment of the filter with ultraviolet light, hybridization was performed. The filter was washed to a final stringency of 0.2% saturated sodium citrate (SSC) and 0.1% sodium lauryly sulfate (SDS) and exposed to film. The human Jh probe has been previously reported.[6]

Genomic library. The genomic library was made using pub-

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lished methods.[5] Approximately 100 μg of high mol wt genomic DNA were partially digested with the *Sau*3A restriction enzyme. Fragments from 9 to 23 kilobases (kb) in size were isolated on a sucrose gradient and ligated into phage EMBL3A (Strategene, San Diego). Recombinant phage were packaged, plated, and screened as previously reported.[5]

DNA sequencing. Fragments for sequencing were cloned into M13 vectors and sequenced by the chain termination method using Sequenase (United States Biochemical, Cleveland).[7] All sequence data were derived from both strands.

RESULTS

We studied a bone marrow sample from a patient with ALL and associated peripheral eosinophilia. Karyotypic analysis showed the characteristic t(5;14)(q31;q32) translocation. These features define a distinctive subtype of ALL.[3,4] The leukemic cells were analyzed for cell surface phenotype by immunofluorecence. They were positive for B1 (CD20), B4 (CD19), cALLA (CD10), HLA-DR, and terminal deoxynucleotidyl transferase (Tdt), but negative for surface immunoglobulin. This phenotypic profile describes an immature cell from the B-lymphocytic lineage.[8]

The leukemia DNA was analyzed by Southern blotting for rearrangements of the IgH gene. Using a human immunoglobulin Jh probe, a single rearranged band was detected by *Eco*RI, *Hind*III, *Sst*I, *Sau*3A, and *Eco*RI plus *Hind*III restriction digests, suggesting rearrangement of one allele (Fig 1). The immunoglobulin Jh region from the other allele was presumably either deleted or in the germline configuration.

We hypothesized that the t(5;14)(q31;q32) juxtaposed a growth-promoting gene on chromosome 5 with the immunoglobulin Jh region on chromosome 14. Therefore, a genomic library was made from the leukemic sample and screened with a Jh probe. Fifteen distinct positive clones were isolated and screened for the presence of the rearranged *Sau*3A fragment that was detected by DNA blotting. By this analysis, five clones appeared to represent the rearranged allele identified by DNA blots. One of these clones (clone no. 4) was chosen for further study and a detailed restriction map was generated. The *Eco*RI, *Hind*III/*Eco*RI, and *Sst*I fragments from clone no. 4 that hybridized to the human Jh probe were also identical in size to the rearranged fragments from the leukemia sample, confirming that clone no. 4 represented the rearranged leukemic allele.

Phage clone no. 4 contained 3.7 kb of unknown origin joined to the IgH gene in the region of Jh4 (Fig 2). The IgH gene from Jh4 to the Cmu region appeared to be in germline configuration. Previously, the gene encoding hematopoietic growth factor IL-3 had been mapped to chromosome 5q31 so it was suspected that clone no. 4 might contain part of this gene.[9-12] When the restriction map of human IL-3 and clone no. 4 were compared, they were identical for more than 3 kb (Fig 2).

We confirmed the juxtaposition of the IL-3 gene and the IgH gene by nucleic acid sequencing of the subcloned *Bst*EII/*Hpa*I fragment (Fig 2). The sequence of this fragment showed no disruption of the protein coding region or the messenger RNA of the IL-3 gene. The break in the IL-3 gene occurred in the promotor region, 452 base pairs (bp) upstream of the transcriptional start site (position 64, Fig
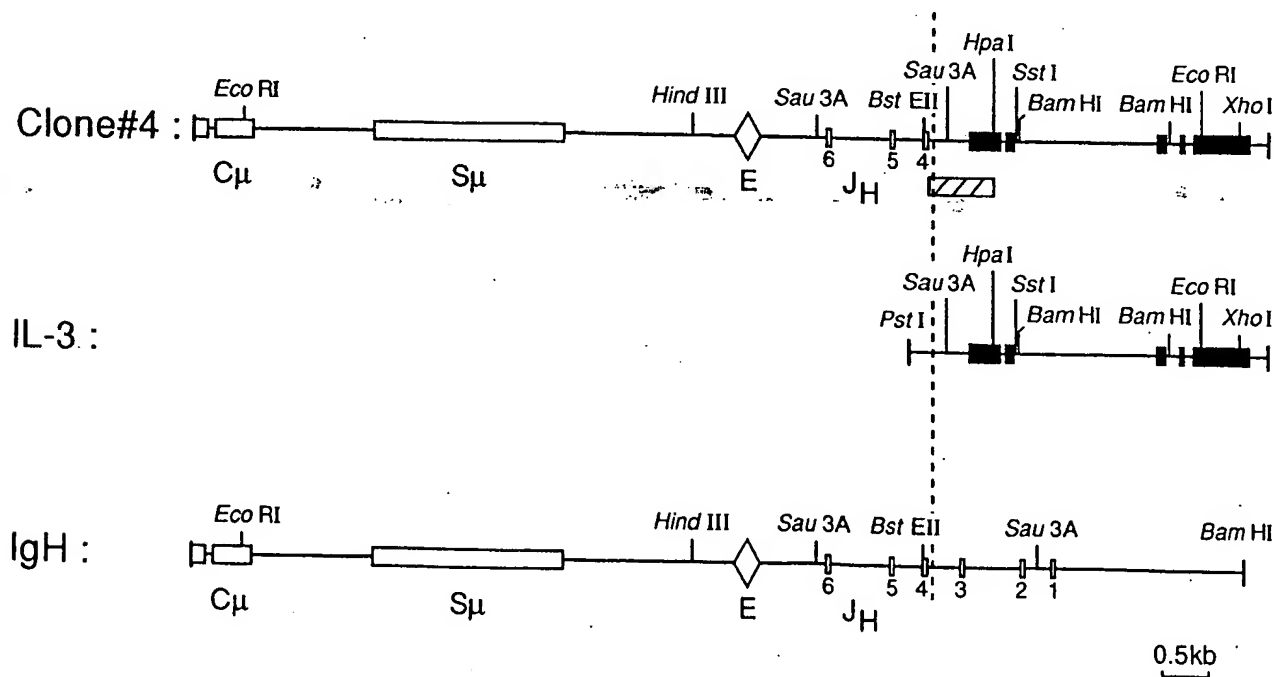
Fig 2. Breakpoint region: t(5;14)(q31;q32). Comparative mapping of phage clone no. 4, the germline IgJh region, and the germline IL-3 gene.[28,29] The map of clone no. 4 is identical to that of IgH until it diverges in the region of Jh4 (at the dashed line), after which it is identical to the map of IL-3. The two genes are positioned in a head-to-head orientation. The Ig μ chain constant region (Cμ), switch region (Sμ), enhancer (E), and Jh segments are indicated (open symbols). The five exons (dark boxes) and four introns of the IL-3 gene are shown. The hatched box indicates the sequenced region.

3A). The break in the IgH gene occurred 2 bp upstream of the Jh4 region. Between the two breaks, 25 bp of uncertain origin (putative N sequence) were inserted.[13,14] No sequences homologous to the immunoglobulin heptamer and nonamer could be identified in the IL-3 sequence (Fig 3B). Therefore, nucleic acid sequencing confirmed the juxtaposition of the IL-3 gene and the IgH gene. The sequence data clearly showed that the genes were positioned in opposite transcriptional orientations (head-to-head).

Available data also allowed us to determine the normal positions of the IL-3 gene and the GM-CSF gene in relation to the centromere of chromosome 5 (Fig 4). The IgH gene is known to be positioned with the variable regions toward the telomere on chromosome 14q.[2,15] It has also been shown that

GM-CSF maps within 9 kb of IL-3 in the same transcriptional orientation.[16] Using this information and assuming a simple translocation event in our sample, we can conclude that the IL-3 gene is normally more centromeric, and the GM-CSF gene more telomeric on chromosome 5q (Fig 4). Furthermore, both are transcribed with their 5' ends toward the centromere.

DISCUSSION

In this report we have cloned a unique chromosomal translocation that appears to be a consistent feature of a rare, yet distinct, clinical form of acute leukemia. This translocation joined the promotor of the IL-3 gene to the IgH gene. Except for the altered promotor, the IL-3 gene appeared
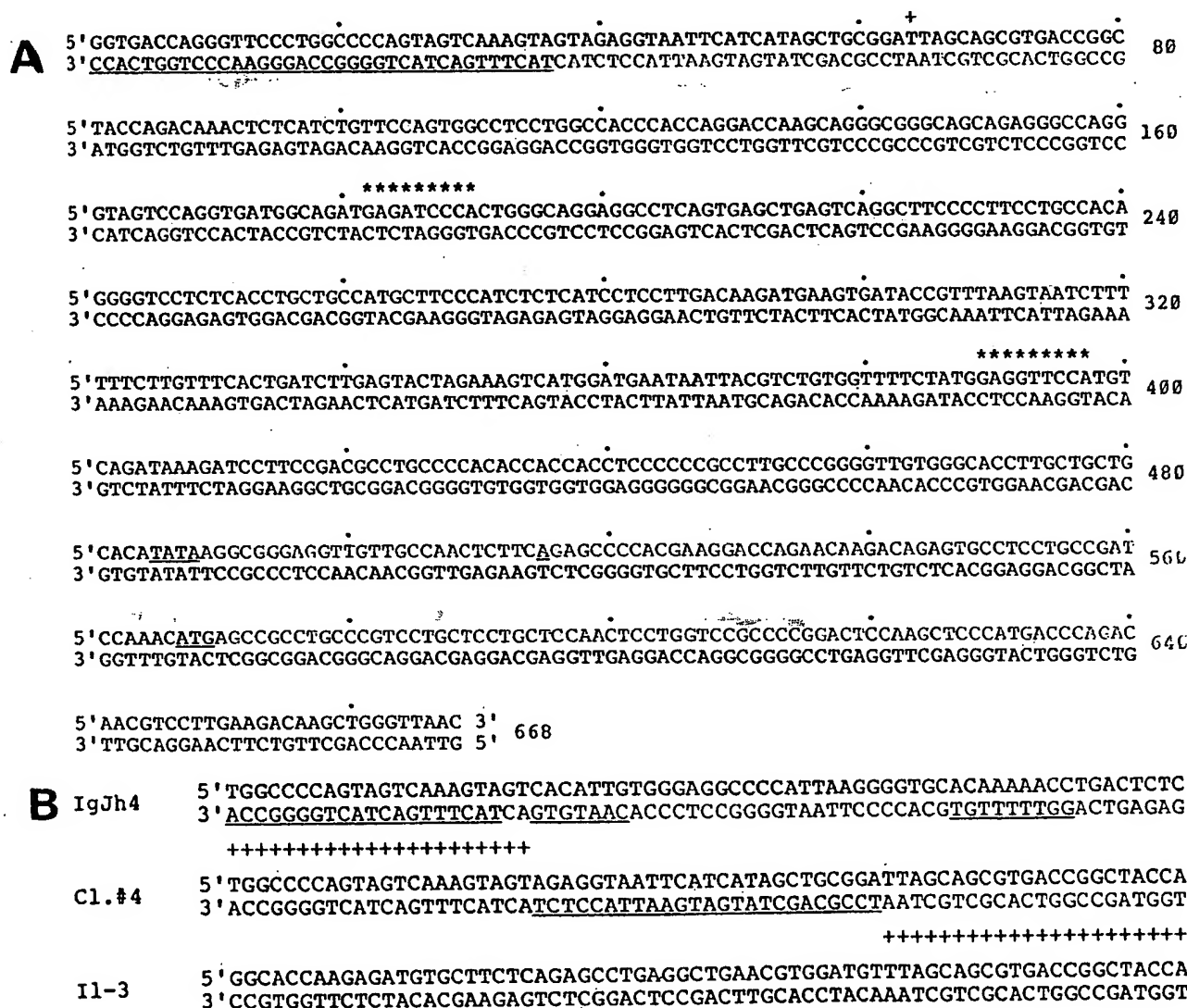
A
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5'GGTGACCAGGGTTCCCTGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGC   80
3'CCACTGGTCCCAAGGGACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCG

5'TACCAGACAAACTCTCATCTGTTCCAGTGGCCTCCTGGCCACCCACCAGGACCAAGCAGGGCGGGCAGCAGAGGGCCAGG  160
3'ATGGTCTGTTTGAGAGTAGACAAGGTCACCGGAGGACCGGTGGGTGGTCCTGGTTCGTCCCGCCCGTCGTCTCCCGGTCC

          *********
5'GTAGTCCAGGTGATGGCAGATGAGATCCCACTGGGCAGGAGGCCTCAGTGAGCTGAGTCAGGCTTCCCCTTCCTGCCACA  240
3'CATCAGGTCCACTACCGTCTACTCTAGGGTGACCCGTCCTCCGGAGTCACTCGACTCAGTCCGAAGGGGAAGGACGGTGT

5'GGGGTCCTCTCACCTGCTGCCATGCTTCCCATCTCTCATCCTCCTTGACAAGATGAAGTGATACCGTTTAAGTAATCTTT  320
3'CCCCAGGAGAGTGGACGACGGTACGAAGGGTAGAGAGTAGGAGGAACTGTTCTACTTCACTATGGCAAATTCATTAGAAA

                                                            *********
5'TTTCTTGTTTCACTGATCTTGAGTACTAGAAAGTCATGGATGAATAATTACGTCTGTGGTTTTCTATGGAGGTTCCATGT  400
3'AAAGAACAAAGTGACTAGAACTCATGATCTTTCAGTACCTACTTATTAATGCAGACACCAAAAGATACCTCCAAGGTACA

5'CAGATAAAGATCCTTCCGACGCCTGCCCCACACCACCACCTCCCCCCGCCTTGCCCGGGGTTGTGGGCACCTTGCTGCTG  480
3'GTCTATTTCTAGGAAGGCTGCGGACGGGGTGTGGTGGTGGAGGGGGGCGGAACGGGCCCCAACACCCGTGGAACGACGAC

5'CACATATAAGGCGGGAGGTTGTTGCCAACTCTTCAGAGCCCCACGAAGGACCAGAACAAGACAGAGTGCCTCCTGCCGAT  560
3'GTGTATATTCCGCCCTCCAACAACGGTTGAGAAGTCTCGGGGTGCTTCCTGGTCTTGTTCTGTCTCACGGAGGACGGCTA

5'CCAAACATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCAACTCCTGGTCCGCCCCGGACTCCAAGCTCCCATGACCCAGAC  640
3'GGTTTGTACTCGGCGGACGGGCAGGACGAGGACGAGGTTGAGGACCAGGCGGGGCCTGAGGTTCGAGGGTACTGGGTCTG

5'AACGTCCTTGAAGACAAGCTGGGTTAAC 3'
3'TTGCAGGAACTTCTGTTCGACCCAATTG 5'   668
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B IgJh4
```
5'TGGCCCCAGTAGTCAAAGTAGTCACATTGTGGGAGGCCCCATTAAGGGGTGCACAAAAACCTGACTCTC
3'ACCGGGGTCATCAGTTTCATCAGTGTAACACCCTCCGGGGTAATTCCCCACGTGTTTTTGGACTGAGAG
       +++++++++++++++++++++++++
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C1.#4
```
5'TGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGCTACCA
3'ACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCGATGGT
                        +++++++++++++++++++++++++
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Il-3
```
5'GGCACCAAGAGATGTGCTTCTCAGAGCCTGAGGCTGAACGTGGATGTTTAGCAGCGTGACCGGCTACCA
3'CCGTGGTTCTCTACACGAAGAGTCTCGGACTCCGACTTGCACCTACAAATCGTCGCACTGGCCGATGGT
```

Fig 3. Sequence of t(5;14)(q31;q32) breakpoint region. (A) Nucleotide sequence of the *Bst*EII/*Hpa*I fragment indicated on Fig 2. Nucleotides 1 to 36 represent the Jh4 coding region underlined on the coding strand. Nucleotides 39 to 63 are a putative N region. The sequence from position 64 to 668 is that of the germline IL-3 gene.[28] The IL-3 TATA box (485), transcription start (515), and initiation methionine (567) are underlined. Two proposed regulatory sequences in the promotor are marked by asterisks (positions 182 and 389). (B) Comparative sequence of the t(5;14)(q31;q32) breakpoint region. The IgJh4 region is shown with its coding region, heptamer, and nonamer underlined. Clone no. 4 is shown with putative N region sequences underlined. The IL-3 sequence is also shown. A plus sign (+) denotes the identical nucleotide between sequences. No heptamer or nonamer is identified in the IL-3 sequence.
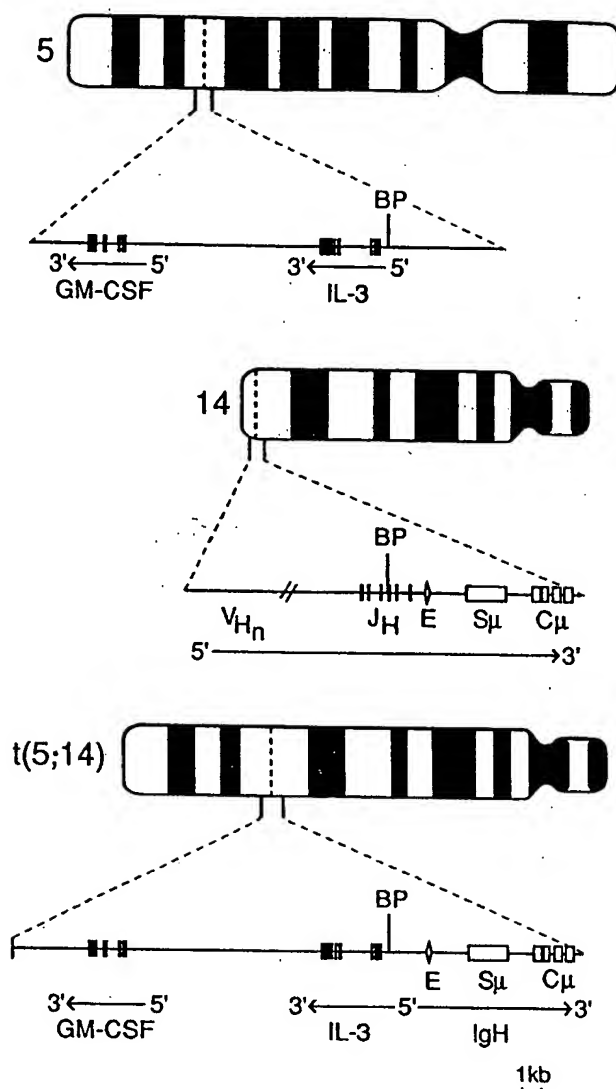
Fig 4. Diagram of the translocation. The normal chromosome 5q31 is shown with the GM-CSF gene telomeric to the IL-3 gene in the transcriptional orientation shown. On normal chromosome 14q32 the Vh regions are telomeric. The t(5;14)(q31;q32) translocation results in the head-to-head orientation of these genes. Symbols are defined in Fig 2. BP, breakpoint position.

intact as no deletions, insertions, or point mutations were detected by restriction mapping of the entire gene and sequencing of part of the gene. The IgH gene has been truncated at the Jh4 region, which places the immunoglobulin enhancer within 2.5 kb of the IL-3 gene.[17,18] This leads to the hypothesis that the enhancer is increasing transcription of a structurally normal IL-3 gene. The same mechanism is important for activation of the c-*myc* gene in some cases of Burkitt's lymphoma.[19] An alternate hypothesis is that the elimination of an upstream IL-3 promotor element is crucial to the activation of the IL-3 gene.

The proposed activation of the IL-3 gene suggests that an autocrine loop is important for the pathogenesis of this leukemia.[20] Over-expression of the IL-3 gene coupled with

the presence of the IL-3 receptor in these cells could account for a strong stimulus for proliferation. In this regard, there are data indicating that immature B-lineage lymphocytes and B-lineage leukemias may express the IL-3 receptor.[21,22]

An additional feature of this type of leukemia is the dramatic eosinophilia, consisting of mature forms. It has been hypothesized that the eosinophils do not arise from the malignant clone, but are stimulated by the tumor.[23,24] Because of the known effect of IL-3 on eosinophil differentiation, secretion of high levels of IL-3 by leukemic cells might have a role in the eosinophilia in this type of leukemia.[12]

The data suggest that the recombination mechanism that is active in the IgH gene during normal differentiation has a role in this translocation.[13,14] This is supported by the breakpoint location at the 5' end of Jh4 and the presence of putative N-region sequences. On the other hand, no recombination signal sequence (heptamer and nonamer) was found in this region on chromosome 5, suggesting that additional factors also played a role. Further studies will elucidate the mechanism of this and other translocations.

In the leukemia we studied, it is possible that the immunoglobulin enhancer also activates the GM-CSF gene, since this gene is probably positioned only 14 kb away (Fig 4). This is known to be within the range of enhancer activation.[25] The interleukin-5 (IL-5) gene maps to chromosome 5q31.[26] Deregulation of the IL-5 gene by this translocation would act synergistically with IL-3 in the stimulation of eosinophil proliferation and differentiation.[27] These and other questions will be answered by the study of more patient samples. We plan to determine whether the t(5;14)(q31;q32) translocation is capable of activating multiple lymphokines simultaneously and whether they cooperate in the generation of this leukemia.

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Activation of the Interleukin-3 Gene by Chromosome Translocation in Acute Lymphocytic Leukemia With Eosinophilia

By Timothy C. Meeker, Dan Hardy, Cheryl Willman, Thomas Hogan, and John Abrams

The t(5;14)(q31;q32) translocation from B-lineage acute lymphocytic leukemia with eosinophilia has been cloned from two leukemia samples. In both cases, this translocation joined the IgH gene and the interleukin-3 (IL-3) gene. In one patient, excess IL-3 mRNA was produced by the leukemic cells. In the second patient, serum IL-3 levels were measured and shown to correlate with disease activity. There was no evidence of excess granulocyte/macrophage colony stimulating factor (GM-CSF) or IL-5 expression. Our data support the formulation that this subtype of leukemia may arise in part because of a chromosome translocation that activates the IL-3 gene, resulting in autocrine and paracrine growth effects.
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A NUMBER OF chromosome translocations have been associated with human leukemia and lymphoma. In many cases the study of these translocations has led to the discovery or characterization of proto-oncogenes, such as bcl-2, c-abl, and c-myc, that are located adjacent to the translocation.[1,2] It is now widely understood that cancer-associated translocations disrupt nearby proto-oncogenes.

A distinct subtype of acute leukemia is characterized by the triad of B-lineage immunophenotype, eosinophilia, and the t(5;14)(q31;q32) translocation.[3,4] Leukemic cells from such patients have been positive for terminal deoxynucleotidyl transferase (Tdt), common acute lymphoblastic leukemia antigen (CALLA), and CD19, but negative for surface or cytoplasmic immunoglobulin. In previous work, we cloned the t(5;14) breakpoint from one leukemic sample (Case 1) and determined that the IgH and interleukin-3 (IL-3) genes were joined by this abnormality.[5] In this report, we extend those findings by showing that the t(5;14)(q31;q32) translocation from a second leukemia sample (Case 2) has a similar structure, and we report our study of growth factor expression in these patients.

MATERIALS AND METHODS

Samples and Southern blots. Case 1 has been described.[5,6] Clinical features of Case 2 have been described in detail.[3] DNA isolation and Southern blotting was done using previously described methods.[5] Filters were hybridized with an immunoglobulin Jh probe, a 280 bp BamHI/EcoRI genomic IL-3 fragment, and an IL-3 cDNA probe.[7,8]

Northern blots. RNA isolation and Northern blotting have been described.[9] Briefly, Northern blots were done by separating 9μg total RNA on 1% agarose-formaldehyde gels. Equal RNA loading in each lane was confirmed by ethidium bromide staining. Blots were hybridized with an IL-3 cDNA probe extending to the Xho I site in exon 5, a 720 bp Sst I/Kpn I probe derived from intron 2 of the IL-3 gene, a 600 bp Nhe I/Hpa I IL-5 cDNA probe, and a 500 bp Pst I/Nco I granulocyte-macrophage colony stimulating factor (GM-CSF) cDNA probe.[10-12]

Polymerase chain reaction. Primers were designed with BamHI sites for cloning. One primer hybridized to the Jh sequences from the IgH gene (Primer 144: 5'-TAGGATCCGACGGTGACCAGGGT), and the other hybridized to the region of the TATA box in the IL-3 gene (Primer 161: 5'-AACAGGATCCCGCCTTATATGTGCAG). Polymerase chain reaction (PCR) (95°C for 1 minute, 61°C for 30 seconds, and 72°C for 3 minutes) was done using 500 ng genomic DNA and 50 pmol of each primer in 100 μL containing 67 mmol/L Tris-HCl pH 8.8, 6.7 mmol/L MgCl$_2$, 10% dimethyl sulfoxide (DMSO), 170 μg/mL bovine serum albumin (BSA) (fraction V),

16.6 mmol/L ammonium sulfate, 1.5 mmol/L each dNTP and Taq polymerase (Perkin-Elmer, Norwalk, CT).[13]

Sequencing. Sequencing was done by chain termination in M13 vectors.[14] As part of this study, we sequenced a subclone of a normal IL-3 promotor, covering 598 base pairs from a Sma I site at position −1240 (with respect to the proposed site of transcription initiation) to an Nhe I site at position −642. The plasmid containing this region was a gift from Naoko Arai of the DNAX Research Institute.

Expression in Cos7 cells. A genomic IL-3 fragment from Case 1 was cloned into the pXM expression vector.[10] Briefly, the HindIII/Sal I fragment containing the IL-3 gene was subcloned from the previously described phage clone 4 into pUC18.[5] The 2.6 kb fragment extending from the Sma I site 61 bp upstream of the IL-3 transcription start to the Sma I site in the polylinker was cloned into the blunted Xho I site of pXM. The negative control construct was the pXM vector without insert. Plasmids were introduced into Cos7 cells by electroporation, and supernatant was collected after 48 hours in culture.

TF1 bioassay. TF-1 cells were passaged in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, and 1 ng/mL human GM-CSF.[15] Samples and antibodies were diluted in this same medium lacking GM-CSF but containing penicillin and streptomycin. A 25 μL volume of serial dilutions of patient serum was added to wells in a flat bottom 96-well microtiter plate. Rat anti-cytokine monoclonal antibody in a volume of 25 μL was added to appropriate wells and preincubated for 1 hour at 37°C. Fifty microliters of twice washed TF-1 cells were added to each well, giving a final cell concentration of 1 × 10^4 cells per well (final volume, 100 μL). The plate was incubated for 48 hours. The remaining cell viability was determined metabolically by the colori-

IgJh5 5'GACGGTGACCAGGGTTCCTTGGCCCCAGGGAGTCGAACCCAGTTGTCACATTGTGACAACAATGCCAGACCCCGACAAAGAACGGGCGCCCCGCACGTCCCT
3'CTGCCACTGGTCCCAAGGAACCGGGGTCCTCAGCTTGGTCAACAGTGTAACACTGTTGTTACGGTCTGGGGCTGTTTCTTGCCCGCGGGGCCGTGCAGGGA

++++++++++++++++++ ++++++++++ +++++++++++++++

Clone 5'GACGGTGACCAGGGTTCCCTGGCCCCAGGGGTCGAACCCAGTTGTACCGCCATGTTATTGGGGGGGATCAAGACCTCTCAATACAACTGTCTCCTGCCAAT
3'CTGCCACTGGTCCCAAGGGACCGGGGTCCCCAGCTTGGTCAACATGGCGGTACAATAACCCCCCCTAGTTCTGGAGAGTTATGTTGACAGAGGACGGTTA

(-934)*++

IL3 5'CCCCTCTCTGCAAACCTTGCCTACTGGGCCTGCACCTGGCAAATCCATGCTCAGCACAGACGGGGATCAAGACCTCTCAATACAACTGTCTCCTGCCAAT
3'GGGGAGAGACGTTTGGAACGGATGACCCGGACGTGGACCGTTTAGGTACGAGTCGTGTCTGCCCCTAGTTCTGGAGAGTTATGTTGACAGAGGACGGTTA

Fig 1. Breakpoint sequences for Case 2. The germline IgJh5 region sequence (protein coding region and recombination signal sequences are underlined) is on top, the translocation sequence from Case 2 (PCR primer sequences and putative N region are underlined) is in the middle, and the germline IL-3 sequence, which we derived from a normal IL-3 clone, is on the bottom.[7] + indicates that each sequence has the same nucleotide. The sequence documents the head-to-head joining of the IL-3 and IgH genes. The breakpoint in the IL-3 gene occurred at position −934 (*).

metric method of Mosmann using a VMax microtiter plate reader (Molecular Devices, Menlo Park, CA) set at 570 and 650 nm.[16]

Cytokine immunoassays. These assays used rat monoclonal anti-cytokine antibodies (10 μg/mL) to coat the wells of a PVC microtiter plate. The capture antibodies used were BVD3-6G8, JES1-39D10, and BVD2-23B6, for the IL-3, IL-5, and GM-CSF assays, respectively. Patient sera were then added (undiluted and diluted 1:2 for IL-3, undiluted for IL-5, and undiluted and diluted 1:5 for GM-CSF). The detecting immunoreagents used were either mouse antiserum to IL-3 or nitroiodophenyl (NIP)-derivatized rat monoclonal antibodies JES1-5A2 and BVD2-21C11, specific for IL-5 and GM-CSF, respectively. Bound antibody was subsequently detected with immunoperoxidase conjugates: horseradish peroxidase (HRP)-labeled goat anti-mouse Ig for IL-3, or HRP-labeled rat (J4 MoAb) anti-NIP for IL-5 and GM-CSF. The chromogenic substrate was 3-3′azino-bis-benzthiazoline sulfonate (ABTS; Sigma, St Louis, MO). Unknown values were interpolated from standard curves prepared from dilutions of the recombinant factors using Softmax software available with the VMAX microplate reader (Molecular Devices).

RESULTS

Leukemic DNA from Case 2 was studied by Southern blotting. When digested with the *Hind*III restriction enzyme and hybridized with a human immunoglobulin heavy chain joining region (Jh) probe, a rearranged fragment at approximately 14 kb was detected (data not shown). When reprobed with either of two different IL-3 probes, a rearranged 14 kb fragment, comigrating with the rearranged Jh fragment, was identified. When leukemic DNA was digested with *Hind*III plus *Eco*RI, a rearranged Jh fragment was detected at 6 kb. The IL-3 probes also identified a comigrating fragment of this size. These experiments indicated that the leukemic sample studied was clonal and that a single fragment contained both Jh and IL-3 sequences, suggesting a translocation had occurred.

To characterize better the joining of the IL-3 gene and the immunoglobulin heavy chain (IgH) gene, the polymerase chain reaction (PCR) was used to clone the translocation.[13] A Jh primer and an IL-3 primer were designed to produce an amplified product in the event of a head-to-head translocation. While control DNA gave no PCR product, Case 2 DNA yielded a PCR-derived fragment of approximately 980 bp, which was cloned and sequenced.

The DNA sequence of the translocation clone from Case 2 confirmed the joining of the Jh region with the promotor of the IL-3 gene in a head-to-head configuration (Fig 1). Sequence analysis indicated that the breakpoint on chromosome 14 was just upstream of the Jh5 coding region. The breakpoint on chromosome 5 occurred 934 bp upstream of the putative site of transcription initiation of the IL-3 gene. We also determined that a putative N sequence of 17 bp was inserted between the chromosome 5 and chromosome 14 sequences during the translocation event.[17,18] Figure 2 shows

Fig 2. Relationship of chromosome 5 breakpoints to the IL-3 gene. This figure shows the two cloned breakpoints (arrows) in relation to the normal IL-3 gene.[8,10] One breakpoint occurred at position −462 and the other at −934 (arrows). In both circumstances, the translocations resulted in a head-to-head joining of the IgH gene and the IL-3 gene, leaving the mRNA and protein coding regions of the IL-3 gene intact. Boxes denote the five IL-3 exons; restriction enzymes are (B) *Bam*HI, (P) *Pst* I, (H) *Hpa* I, (E) *Eco*RI, and (X) *Xho* I.
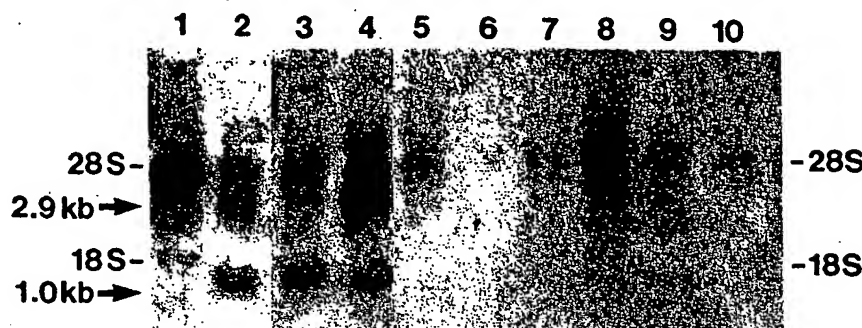
Fig 3. Documentation of IL-3 mRNA over-expression. A Northern blot was prepared and hybridized with a probe for IL-3. Lane 1 contained RNA from unstimulated peripheral blood lymphocytes (PBL) as a negative control. Lane 2 contained RNA from PBL stimulated for 4 hours with concanavalian A (ConA), and lane 3 contained RNA from PBL stimulated with ConA for 48 hours. As in the positive control lanes (2 and 3), a 1 kb band was identified in the leukemic sample from Case 1 (lane 4, lower arrow), suggesting aberrant expression of the IL-3 gene. In addition, the leukemic sample showed over-expression of an unspliced 2.9 kb IL-3 transcript (lane 4, upper arrow). We documented that this represented an unspliced precursor of the mature 1 kb transcript by showing that this band hybridized to a probe from intron 2 of the IL-3 gene. A similar 2.9 kb band was detected in lane 2, suggesting that an IL-3 mRNA of this size is sometimes detectable in normal mitogen-stimulated cells. Lane 5 through 10 represent RNA from six samples of B-lineage acute lymphocytic leukemia without the t(5;14) translocation, indicating that only the sample with the translocation exhibited IL-3 over-expression. Case 2 could not be analyzed by Northern blot because too few cells were available for study.

the locations of the two cloned breakpoints in relation to the IL-3 gene. The two chromosome 5 breakpoints were separated by less than 500 bp.

The genomic structure in Cases 1 and 2 suggested that a normal IL-3 gene product was over-expressed as a result of the altered promotor structure. This would predict that the IL-3 gene on the translocated chromosome was capable of making IL-3 protein. This prediction was tested by expressing a genomic fragment from the translocated allele of Case 1 containing all five IL-3 exons under the control of the SV40 promotor/enhancer in the Cos7 cell line. Cell supernatants were studied in a proliferation assay using the factor dependent erythroleukemic cell line, TF-1. The supernatants derived from transfections using the vector plus insert supported TF-1 proliferation, while supernatants from transfections using the vector alone were negative in this assay (data not shown). Furthermore, the biologic activity could be blocked by an antibody to human IL-3 (BVD3-6G8). This result showed that the translocated allele retained the ability to make IL-3 mRNA and protein.

The level of expression of IL-3 mRNA in leukemic cells from Case 1 was assessed. Northern blotting showed that the mature IL-3 mRNA (approximately 1 kb) and a 2.9 kb unspliced IL-3 mRNA were excessively produced by the leukemia (Fig 3). The 2.9 kb form of the mRNA is also present at low levels in normal peripheral blood T lymphocytes after mitogen activation (Fig 3). Several B-lineage acute leukemia samples without the t(5;14) translocation had undetectable levels of IL-3 mRNA in these experiments. In addition, although genes for GM-CSF and IL-5 map close to the IL-3 gene and might have been deregulated by the translocation, no IL-5 or GM-CSF mRNA could be detected in the leukemic sample (data not shown).[19,20]

Three serum samples from Case 2 were assayed by immunoassay for levels of IL-3, GM-CSF, and IL-5 (Table 1). Serum IL-3 could be detected and correlated with the clinical course. When the patient's leukemic cell burden was highest, the IL-3 level was highest. No serum GM-CSF or IL-5 could be detected.

Since the IL-3 immunoassay measured only immunoreactive factor, we confimed that biologically active IL-3 was present by using the TF-1 bioassay. This bioassay can be rendered monospecific using appropriate neutralizing monoclonal antibodies specific for IL-3, IL-5, or GM-CSF. We observed that sera from 1-16-84 and 3-14-84 contained TF-1 stimulating activity that could be blocked with anti–IL-3 MoAb (BVD3-6G8), but not with MoAbs to IL-5 (JES1-39D10) or GM-CSF (BVD2-23B6) (Fig 4; GM-CSF data not shown). The amount of neutralizable bioactivity in these two samples correlated very well with the difference in IL-3 levels obtained by immunoassay for these samples. Furthermore, the failure to block TF-1 proliferating activity with either anti–IL-5 or anti–GM-CSF was consistent with the inability to measure these factors by immunoassay and

Table 1. Peripheral Blood Counts and Growth Factor Levels at Different Times in Case 2

	Sample Date		
	11/15/83	1/16/84	3/14/84
Peripheral blood counts (cells/μL)			
WBC	81,800	116,500	12,300
Lymphoblasts	0	33,785	0
Eosinophils	46,626	73,080	615
Serum growth factor levels (pg/mL)			
IL-3	<444	7,995	1,051
GM-CSF	<15	<15	<15
IL-5	<50	<50	<50

Peripheral blood counts from Case 2 at three different time points with the corresponding growth factor levels quantified by immunoassay. The patient received chemotherapy between 1/16/84 and 3/14/84 to lower his leukemic burden.[3] No serum samples were available for a similar analysis of Case 1.

Abbreviation: WBC, white blood cells.
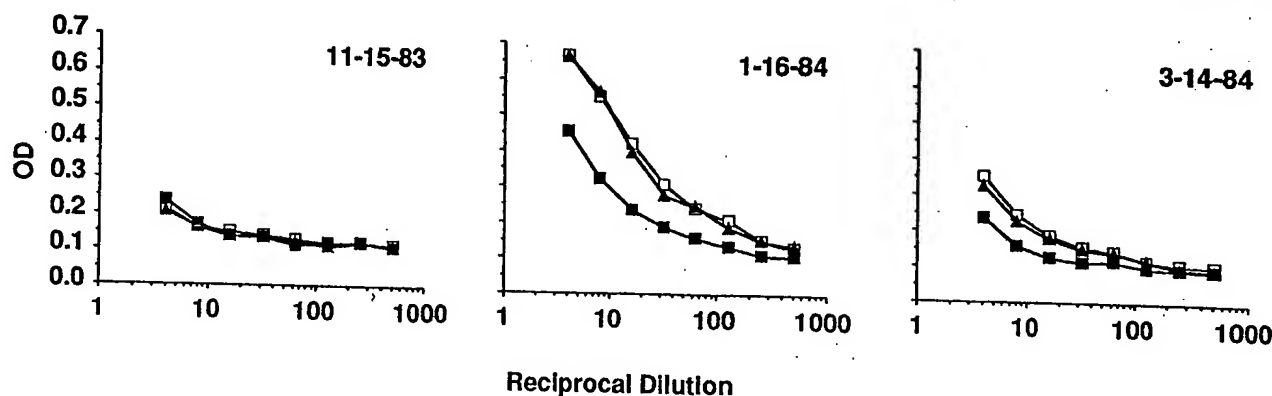
Reciprocal Dilution

Fig 4. Bioassay of serum IL-3. Leukemic patient sera were tested for bioactive IL-3 and IL-5 in the TF-1 proliferation assay. The reciprocal of the dilution is indicated on the horizontal axis and the optical density indicating the amount of proliferation is indicated on the vertical axis. Serum from all three time points was assayed simultaneously. The assay was rendered monospecific by using a 1 µg/mL final concentration of monoclonal rat anti–IL-3, BVD3-6G8 (■), or anti–IL-5, JES1-39D10 (▲); □ indicates no MoAb. On 1/16/84 and 3/14/84, inhibition of proliferation was evident in the presence of anti–IL-3 antibody, documenting serum levels of IL-3 on those days. Serum IL-5 was not detected in this assay, as anti–IL-5 did not alter TF-1 proliferation.

indicated that these other myeloid growth factors were not detectably circulating in the serum of this patient.

DISCUSSION

In this report, we have extended our analysis of acute lymphocytic leukemia and eosinophilia associated with the t(5;14) translocation. In both cases we have studied, we have documented the joining of the IL-3 gene from chromosome 5 to the IgH gene from chromosome 14. The breakpoints on chromosome 5 are within 500 bp of each other, suggesting that additional breakpoints will be clustered in a small region of the IL-3 promotor. The PCR assay we have developed will be useful in the screening of additional clinical samples for this abnormality.

The finding of a disrupted IL-3 promotor associated with an otherwise normal IL-3 gene implied that this translocation might lead to the over-expression of a normal IL-3 gene product. In this work, we have documented that this is true. In addition, neither GM-CSF nor IL-5 are over-expressed by the leukemic cells. Furthermore, in one patient, serum IL-3 could be measured and correlated with disease activity. To our knowledge, this is the first measurement of human IL-3 in serum and its association with a disease process. The measurement of serum IL-3 in this and other clinical settings may now be indicated.

The finding of the IL-3 gene adjacent to a cancer-associated translocation breakpoint suggests that its activation is important for oncogenesis. It is our thesis that an autocrine loop for IL-3 is important for the evolution of this leukemia.[21] The excessive IL-3 production that we have documented would be one feature of such an autocrine loop. The final proof of our thesis must await additional data. In particular, from the study of additional clinical samples, it will be necessary to document that the IL-3 receptor is present on the leukemic cells and that anti–IL-3 antibody decreases proliferation of the leukemia in vitro.

An important aspect of this work is the suggestion of a therapeutic approach for this disease. If an autocrine loop for IL-3 can be documented in this disease, attempts to lower circulating IL-3 levels or block the interaction of IL-3 with its receptor may prove useful. Because it is also possible that the eosinophilia in these patients is mediated by the paracrine effects of leukemia-derived IL-3, similar interventions may improve this aspect of the disease. Antibodies or engineered ligands to accomplish these goals may soon be available.

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Clinical and Pathologic Significance of the c-erbB-2 (HER-2/neu) Oncogene

Timothy P. Singleton and John G. Strickler

The c-erbB-2 oncogene was first shown to have clinical significance in 1987 by Slamon et al,[79] who reported that c-erbB-2 DNA amplification in breast carcinomas correlated with decreased survival in patients with metastasis to axillary lymph nodes. Subsequent studies, however, of c-erbB-2 activation in breast carcinoma reached conflicting conclusions about its clinical significance. This oncogene also has been reported to have clinical and pathologic implications in other neoplasms. Our review summarizes these various studies and examines the clinical relevance of c-erbB-2 activation, which has not been emphasized in recent reviews.[37,38,55] The molecular biology of the c-erbB-2 oncogene has been extensively reviewed[37,38,55] and will be discussed only briefly here.

BACKGROUND

The c-erbB-2 oncogene was discovered in the 1980s by three lines of investigation. The neu oncogene was detected as a mutated transforming gene in neuroblastomas induced by ethylnitrosurea treatment of fetal rats.[8,73,74,78] The c-erbB-2 was a human gene discovered by its homology to the retroviral gene v-erbB.[33,49,76] HER-2 was isolated by screening a human genomic DNA library for homology with v-erbB.[24] When the DNA sequences were determined subsequently, c-erbB-2, HER-2, and neu were found to represent the same gene. Recently, the c-erbB-2 oncogene also has been referred to as NGL.

The c-erbB-2 DNA is located on human chromosome 17q21[24,33,68] and codes for c-erbB-2 mRNA (4.6 kb), which translates c-erbB-2 protein (p185). This

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protein is a normal component of cytoplasmic membranes. The c-erbB-2 oncogene is homologous with, but not identical to, c-erbB-1, which is located on chromosome 7 and codes for the epidermal growth factor receptor.[8,103] The c-erbB-2 protein is a receptor on cell membranes and has intracellular tyrosine kinase activity and an extracellular binding domain.[2,105] Electron microscopy with a polyclonal antibody detects c-erbB-2 immunoreactivity on cytoplasmic membranes of neoplasms, especially on microvilli and the non-villous outer cell membrane.[61] In normal cells, immunohistochemical reactivity for c-erbB-2 is frequently present at the basolateral membrane or the cytoplasmic membrane's brush border.[22,62]

There is experimental evidence that c-erbB-2 protein may be involved in the pathogenesis of breast neoplasia. Overproduction of otherwise normal c-erbB-2 protein can transform a cell line into a malignant phenotype.[25] Also, when the *neu* oncogene containing an activating point mutation is placed in transgenic mice with a strong promoter for increased expression, the mice develop multiple independent mammary adenocarcinomas.[18,63] In other experiments, monoclonal antibodies against the *neu* protein inhibit the growth (in nude mice) of a *neu*-transformed cell line,[26-28] and immunization of mice with *neu* protein protects them from subsequent tumor challenge with the *neu*-transformed cell line.[14] Some authors have speculated that the use of antagonists for the unknown ligand could be useful in future chemotherapy.[85] Further review of this experimental evidence is beyond the scope of this article.

The c-erbB-2 activation most likely occurs at an early stage of neoplastic development. This hypothesis is supported by the presence of c-erbB-2 activation in both in situ and invasive breast carcinomas. In addition, studies of metastatic breast carcinomas usually demonstrate uniform c-erbB-2 activation at multiple sites in the same patient,[11,12,39,41,52] although c-erbB-2 activation has rarely been detected in metastatic lesions but not in the primary tumor.[57,69,107] Even more rarely, c-erbB-2 DNA amplification has been detected in a primary breast carcinoma but not in its lymph node metastasis.[5] In patients who have bilateral breast neoplasms, both lesions have similar patterns of c-erbB-2 activation, but only a few such cases have been studied.[11]

MECHANISMS OF c-erbB-2 ACTIVATION

The most common mechanism of c-erbB-2 activation is genomic DNA amplification, which almost always results in overproduction of c-erbB-2 mRNA and protein.[17,34,65,81] The c-erbB-2 amplification may stabilize the overproduction of mRNA or protein through unknown mechanisms. Human breast carcinomas with c-erbB-2 amplification contain 2 to 40 times more c-erbB-2 DNA[4,5] and 4 to 128 times more c-erbB-2 mRNA[34,90] than found in normal tissue. Most human breast carcinomas with c-erbB-2 amplification have 2 to 15 times more c-erbB-2 DNA. Tumors with greater amplification tend to have greater overproduction.[17,52,65] The non-mammary neoplasms that have been studied tend to have

similar levels of c-erbB-2 amplification or overproduction relative to the corresponding normal tissue.

The second most common mechanism of c-erbB-2 activation is overproduction of c-erbB-2 mRNA and protein without amplification of c-erbB-2 DNA.[81] The quantities of mRNA and protein usually are less than those in amplified cases and may approach the small quantities present in normal breast or other tissues.[17,50,52] The c-erbB-2 protein overproduction without mRNA overproduction or DNA amplification has been described in a few human breast carcinoma cell lines.[47]

Other rare mechanisms of c-erbB-2 activation have been reported. Translocations involving the c-erbB-2 gene have been described in a few mammary and gastric carcinomas, although some reported cases may represent restriction fragment length polymorphisms or incomplete restriction enzyme digestions that mimic translocations.[31,65,75,84,90,108] A single point mutation in the transmembrane portion of neu has been described in rat neuroblastomas induced by ethylnitrosurea.[9,55] The mutated neu protein has increased tyrosine kinase activity and aggregates at the cell membrane.[10,83,98] Although there has been speculation that some of the amplified c-erbB-2 genes may contain point mutations,[46] none has been detected in primary human neoplasms.[41,53,81]

TECHNIQUES FOR DETECTING c-erbB-2 ACTIVATION

Detection of c-erbB-2 DNA Amplification

Amplification of c-erbB-2 DNA is usually detected by DNA dot blot or Southern blot hybridization. In the dot blot method, the extracted DNA is placed directly on a nylon membrane and hybridized with a c-erbB-2 DNA probe. In the Southern blot method, the extracted DNA is treated with a restriction enzyme, and the fragments are separated by electrophoresis, transferred to a nylon membrane, and hybridized with a c-erbB-2 DNA probe. In both techniques, c-erbB-2 amplification is quantified by comparing the intensity (measured by densitometry) of the hybridization bands from the sample with those from control tissue.

Several technical problems may complicate the measurement of c-erbB-2 DNA amplification. First, the extracted tumor DNA may be excessively degraded or diluted by DNA from stromal cells.[81] Second, the c-erbB-2 DNA probe must be carefully chosen and labeled. For example, oligonucleotide c-erbB-2 probes may not be sensitive enough for measuring a low level of c-erbB-2 amplification, because diploid copy numbers can be difficult to detect (unpublished data). Third, the total amounts of DNA in the sample and control tissue must be compensated for, often with a probe to an unamplified gene. Many studies have used control probes to genes on chromosome 17, the location of c-erbB-2, to correct for possible alterations in chromosome number. Identical results, however, are obtained by using control probes to genes on other chromosomes,[5,65,80] with rare exception.[17] Studies using control probes to the beta-

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globin gene must be interpreted with caution, because one allele of this gene is deleted occasionally in breast carcinomas.[3]

Amplification of c-*erb*B-2 DNA was assessed by using the polymerase chain reaction (PCR) in one recent study.[32] Oligoprimers for the c-*erb*B-2 gene and a control gene are added to the sample's DNA, and PCR is performed. If the sample contains more copies of c-*erb*B-2 DNA than of the control gene, the c-*erb*B-2 DNA is replicated preferentially.

Detection of c-*erb*B-2 mRNA Overproduction

Overproduction of c-*erb*B-2 mRNA usually is measured by RNA dot blot or Northern blot hybridization. Both techniques require extraction of RNA but otherwise are analogous to DNA dot blot and Southern blot hybridization. Use of PCR for detection of c-*erb*B-2 mRNA has been described in two recent abstracts.[88,102]

Overproduction of c-*erb*B-2 mRNA can be measured by in situ hybridization. Sections are mounted on glass slides, treated with protease, hybridized with a radiolabeled probe, washed, treated with nuclease to remove unbound probe, and developed for autoradiography. Silver grains are seen only over tumor cells that overproduce c-*erb*B-2 mRNA. Negative control probes are used.[65,96,106] Our experience indicates that these techniques are relatively insensitive for detecting c-*erb*B-2 mRNA overproduction in routinely processed tissue. Although the sensitivity may be increased by modifications that allow simultaneous detection of c-*erb*B-2 DNA and mRNA, in situ hybridization still is cumbersome and expensive (unpublished data).

All of the above c-*erb*B-2 mRNA detection techniques have several problems that make them more difficult to perform than techniques for detecting DNA amplification. One major problem is the rapid degradation of RNA in tissue that is not immediately frozen or fixed. In addition, during the detection procedure, RNA can be degraded by RNase, a ubiquitous enzyme, which must be eliminated meticulously from laboratory solutions. Third, control probes to genes that are uniformly expressed in the tissue of interest need to be carefully selected.

Detection of c-*erb*B-2 Protein Overproduction

The most accurate methods for detecting c-*erb*B-2 protein overproduction are the Western blot method and immunoprecipitation. Both techniques can document the binding specificity of various antibodies against c-*erb*B-2 protein. In Western blot studies, protein is extracted from the tissue, separated by electrophoresis (according to size), transferred to a membrane, and detected by using antibodies to c-*erb*B-2. In immunoprecipitation studies, antibodies against c-*erb*B-2 are added to a tumor lysate, and the resulting protein–antibody precipitate is separated by gel electrophoresis and stained for protein. Both Western blot and immunoprecipitation are useful research tools but currently are not practical for diagnostic pathology. Two recent abstracts have described an enzyme-linked immunosorbent assay (ELISA) for detection of c-*erb*B-2 protein.[19,45]

Overproduction of c-erbB-2 protein is most commonly assessed by various immunohistochemical techniques. These procedures often generate conflicting results, which are explained at least partially by three factors. First, various studies have used different polyclonal and monoclonal antibodies. Because some polyclonal antibodies recognize weak bands in addition to the c-erbB-2 protein band on Western blot or immunoprecipitation, the results of these studies should be interpreted with caution.[22,35,47,61] Even some monoclonal antibodies immunoprecipitate protein bands in addition to c-erbB-2 (p185).[30,59,86] Second, tissue fixation contributes to variability between studies. For example, some antibodies detect c-erbB-2 protein only in frozen tissue and do not react in fixed tissue. In general, formalin fixation diminishes the sensitivity of immunohistochemical methods and decreases the number of reactive cells.[81,86] When Bouin's fixative is used, there may be a higher percentage of positive cases.[22] Third, minimal criteria for interpreting immunohistochemical staining are generally lacking. Although there is general agreement that distinct crisp cytoplasmic membrane staining is diagnostic for c-erbB-2 activation in breast carcinoma, the number of positive cells and the staining intensity required to diagnose c-erbB-2 protein overproduction varies from study to study and from antibody to antibody. Degradation of c-erbB-2 protein is not a problem because it can be detected in intact form more than 24 hours after tumor resection without fixation or freezing.[84]

ACTIVATION OF c-erbB-2 IN BREAST LESIONS

Incidence of c-erbB-2 Activation

Most studies of c-erbB-2 oncogene activation do not specify histological subtypes of infiltrating breast carcinoma. Amplification of c-erbB-2 DNA was found in 19.1 percent (519 of 2715) of invasive carcinomas in 25 studies (Table 1), and c-erbB-2 mRNA or protein overproduction was detected in 20.9 percent (566 of 2714) of invasive carcinomas in 20 studies. Twelve studies have documented c-erbB-2 mRNA or protein overproduction in 15 percent (88 of 604) of carcinomas that lacked c-erbB-2 DNA amplification.

The incidence of c-erbB-2 activation in infiltrating breast carcinoma varies with the histological subtype. Approximately 22 percent (142 of 650) of infiltrating ductal carcinomas have c-erbB-2 activation, as expected from the above data. Other variants of breast carcinoma with frequent c-erbB-2 activation are inflammatory carcinoma (62 percent, 54 of 87), Paget's disease (82 percent, 9 of 11), and medullary carcinoma (22 percent, 5 of 23). In contrast, c-erbB-2 activation is infrequent in infiltrating lobular carcinoma (7 percent, 5 of 73) and tubular carcinoma (7 percent, 1 of 15).

The c-erbB-2 protein overproduction is present in 44 percent (44 of 100) of ductal carcinomas in situ and especially comedocarcinoma in situ (68 percent, 49 of 72). The micropapillary type of ductal carcinoma in situ also tends to have c-erbB-2 activation,[40,54,68] especially if larger cells are present. The greater fre-

TABLE 1. *c-erbB-2* ACTIVATION IN MALIGNANT HUMAN BREAST NEOPLASMS

Histological Diagnosis	c-erbB-2 DNA Amplification[a]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[b]
Carcinoma, not otherwise specified	146/526,[81] 52/310,[17] 52/291,[109] 28/176,[67] 17/157,[113] 22/141,[35] 14/136,[57] 12/122,[1] 19/103,[79] 15/95,[90] 15/86,[111] 17/73,[7] 16/66,[42] 6/61,[50] 11/57,[52] 10/57,[65] 13/51,[13] 8/49,[21] 10/38,[92] 12/35,[94] 1/25,[15] 7/24,[91] 7/15,[31] 7/10,[98] 2/10[107]	42/180,[59] 49/126,[36] 19/62,[65] 19/57,[50] 3/11,[90] 6/10,[96] 3/9[31]	118/728,[65b] 58/330,[17b] 47/313,[65] 17/195,[11] 32/191,[58] 31/185,[101] 34/102,[42] 24/53,[52b] 23/47,[13] 22/45,[9] 11/36,[64] 7/24,[91] 1/10[51]
Carcinoma, type not specified but lacking c-erbB-2 DNA amplification	—	18/136,[81] 14/73,[34] 8/16,[65] 0/8,[90] 1/4,[31] 0/3[96]	16/231,[17b] 18/136,[81] 13/35,[13] 14/29,[52b] 1/28,[92] 3/24,[94] 0/17[91]
Infiltrating ductal carcinoma	21/118,[92] 23/107,[34] 17/50,[44] 7/37[90] 14/53 (comedo-carcinoma)[16] 3/33 (tubuloductal carcinoma)[16]	35/95[34]	22/137,[40] 14/93,[39] 9/34[69]

Inflammatory carcinoma	33/80,[35] 3/6[52]	46/75[35]	5/6[52b]
Paget's disease	—		5/6,[40] 2/3,[54] 2/2[92]
Tubular carcinoma	0/5,[16] 0/1[33]	0/1[34]	1/9[40]
Medullary carcinoma	2/4,[16] 0/1[34]		1/12,[40] 1/3,[58] 1/2,[92]
			0/1[39]
Mucinous carcinoma	0/1,[16] 0/1[53]		1/2[88]
	0/2[88]		
Invasive papillary carcinoma			—
Infiltrating lobular carcinoma	1/15,[16] 0/6[34]	1/5[54]	2/27,[92] 0/12,[40] 0/9,[38]
			1/5[88]
Mammary fibrosarcoma	0/1[33]		—
"Benign cystosarcoma"	3/5[92]	?	0/1[88]
Ductal CIS[c] with minimal invasion		1/2[34]	—
Ductal CIS	0/2[34]		33/74,[40] 10/24[39]
Ductal CIS, solid or comedo type	—		20/33,[58] 19/29,[92]
			10/10[54]
Ductal CIS, micropapillary type	—		10/10[88]
Ductal CIS, micropapillary or cribriform type	—		1(focal)/14[54]
Ductal CIS, papillary or cribriform type	—		0/16,[92] 1/9,[88] 0/3[40]
Lobular CIS	—		0/16[40]

[a] Shown as number of cases with activation/number of cases studied; reference is given as a superscript.

[b] These protein studies used Western blots; the rest used immunohistochemical methods.

[c] CIS = carcinoma in situ.

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quency of c-erbB-2 protein overproduction in comedocarcinoma in situ, compared with infiltrating ductal carcinoma, could be explained by the fact that many infiltrating ductal carcinomas arise from other types of intraductal carcinoma, which show c-erbB-2 activation infrequently. Others have speculated that carcinoma in situ with c-erbB-2 activation tends to regress or to lose c-erbB-2 activation during progression to invasion.[40,68,92] Infiltrating and in situ components of ductal carcinoma, however, usually are similar with respect to c-erbB-2 activation,[11,39] although some authors have noted more heterogeneity of the immunohistochemical staining pattern in invasive than in in situ carcinoma.[40,42,88] Activation of c-erbB-2 is infrequent in lobular carcinoma in situ. If lesions contain more than one histological pattern of carcinoma in situ, the c-erbB-2 protein overproduction tends to occur in the comedocarcinoma in situ but may include other areas of carcinoma in situ.[42,54,68] Overproduction of c-erbB-2 protein in ductal carcinoma in situ correlates with larger cell size and a periductal lymphoid infiltrate.[68]

Activation of c-erbB-2 has not been identified in benign breast lesions, including fibrocystic disease, fibroadenomas, and radial scars (Table 2). Strong membrane immunohistochemical reactivity for c-erbB-2 has not been described in atypical ductal hyperplasia, although weak accentuation of membrane staining has been noted infrequently.[39,42,54] In normal breast tissue, c-erbB-2 DNA is diploid, and c-erbB-2 is expressed at lower levels than in activated tumors.[34,35,65,88]

These preliminary data suggest that c-erbB-2 activation may not be useful for resolving many of the common problems in diagnostic surgical pathology. For example, c-erbB-2 activation is infrequent in tubular carcinoma and radial scars. In addition, because c-erbB-2 activation is unusual in atypical ductal hyperplasia, cribriform carcinoma in situ, and papillary carcinoma in situ, detection of c-erbB-2 activation in these lesions may not be helpful in their differential diagnosis. The histological features of comedocarcinoma in situ, which commonly overproduces c-erbB-2, are unlikely to be mistaken for those of benign lesions. Activation of

TABLE 2. c-erbB-2 ACTIVATION IN BENIGN HUMAN BREAST LESIONS

Histological Diagnosis	c-erbB-2 DNA Amplification[a]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction
Fibrocystic disease	0/10[33]	—	0/32,[39] 0/9,[68] 0/8[85]
Atypical ductal hyperplasia	—	—	2(weak)/21,[54] 1(cytoplasmic)/13[39]
Benign ductal hyperplasia	—	—	0/12[39]
Sclerosing adenosis	—	—	0/4[39]
Fibroadenomas	0/16,[34] 0/6,[93] 0/2,[21] 0/1[91]	0/6,[35] 0/3[34]	0/21,[39] 0/10,[88] 0/8,[88] 0/3[42]
Radial scars	—	—	0/22[39]
Blunt duct adenosis	—	—	0/14[39]
"Breast mastosis"	—	0/3[35]	—

[a]Shown as number of cases with activation/number of cases studied; reference is given as a superscript.

c-*erb*B-2, however, does favor infiltrating ductal carcinoma over infiltrating lobular carcinoma. Further studies of these issues would be useful.

Correlation of c-*erb*B-2 Activation With Pathologic Prognostic Factors

Multiple studies have attempted to correlate c-*erb*B-2 activation with various pathologic prognostic factors (Table 3). Activation of c-*erb*B-2 was correlated with lymph node metastasis in 8 of 28 series, with higher histological grade in 6 of 17 series, and with higher stage in 4 of 14 series. Large tumor size was not associated with c-*erb*B-2 activation in most studies (11 of 14). Tetraploid DNA content and low proliferation, measured by Ki-67, have been suggested as prognostic factors and may correlate with c-*erb*B-2 activation.[6,7]

Correlation of c-*erb*B-2 Activation With Clinical Prognostic Factors

Various studies have attempted also to correlate c-*erb*B-2 activation with clinical features that may predict a poor outcome (Table 4). Activation of c-*erb*B-2 correlated with absence of estrogen receptors in 10 of 28 series and with absence of progesterone receptors in 6 of 18 series. In most studies, patient age did not correlate with c-*erb*B-2 activation, and, in the rest of the reports, c-*erb*B-2 activation was associated with either younger or older ages.

Correlation of c-*erb*B-2 Activation With Patient Outcome

Slamon et al[79,81] first showed that amplification of the c-*erb*B-2 oncogene independently predicts decreased survival of patients with breast carcinoma. The correlation of c-*erb*B-2 amplification with poor outcome was nearly as strong as the correlation of number of involved lymph nodes with poor outcome. Slamon et al also reported that c-*erb*B-2 amplification is an important prognostic indicator only in patients with lymph node metastasis.[79,81]

A large number of subsequent studies also attempted to correlate c-*erb*B-2 activation with prognosis (Table 5). In 12 series, there was a correlation between c-*erb*B-2 activation and tumor recurrence or decreased survival. In five of these series, the predictive value of c-*erb*B-2 activation was reported to be independent of other prognostic factors. In contrast, 18 series did not confirm the correlation of c-*erb*B-2 activation with recurrence or survival. Four possible explanations for this controversy are discussed below.

One problem is that c-*erb*B-2 amplification correlates with prognosis mainly in patients with lymph node metastasis. As summarized in Table 5, most studies of patients with axillary lymph node metastasis showed a correlation of c-*erb*B-2 activation with poor outcome. In contrast, most studies of patients without axillary metastasis have not demonstrated a correlation with patient outcome. Table 6 summarizes the studies in which all patients (with and without axillary metastasis) were considered as one group. There is a trend for studies with a higher percentage of metastatic cases to show an association between c-*erb*B-2 activation and poor outcome. Thus, most of the current evidence suggests that c-*erb*B-2 activation has prognostic value only in patients with metastasis to lymph nodes.

TABLE 3. CORRELATION OF c-erbB-2 ACTIVATION WITH PATHOLOGIC PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	P^a	c-erbB-2 DNA Amplification[b]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[c]
Metastasis to axillary lymph nodes	<0.05	(118)[35] (105)[34] (49)[21] (103)[79] (86)[79] (58)[111]	(104)[35] (92)[34] (9)[31]	(350)[85c] (36)[13] (189)[92]
	0.05–0.15	(279)[17] (176)[57] (157)[113]	(50)[50]	(329)[17c] (261)[84] (195)[111]
	>0.15	(122)[4] (85)[90] (50)[52] (50)[44] (47)[13] (41)[93]	—	(185)[101] (102)[39] (50)[820]
Larger size	<0.05	(280)[17] (86)[79] (176)[57] (157)[113] (103)[79] (64)[77] (58)[111] (45)[21]	—	(330)[17c] (189)[92]
	0.05–0.15		—	(350)[85c] (185)[101] (34)[92]
	>0.15		(51)[50]	—
Higher stage	<0.05	(300)[17] (64)[77] (58)[111] (56)[52]	—	(349)[17a]
	0.05–0.15	(176)[57] (157)[113] (84)[90] (61)[50] (53)[21] (52)[57] (41)[93]	—	(102)[39] (56)[820]
	>0.15		—	—
Higher histological grade	<0.05	(47)[13] (15)[31]	(53)[85]	(176)[101] (168)[11] (38)[13]
	0.05–0.15	(122)[4] (113)[34] (95)[90] (58)[111] (50)[44] (41)[93]	(86)[35] (65)[85]	(290)[85] (189)[92] (102)[39]
	>0.15			—

[a] A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15.
[b] Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.
[c] By Western blot method; all other protein studies used immunohistochemical methods.

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TABLE 4. CORRELATION OF c-erbB-2 ACTIVATION WITH CLINICAL PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	P[a]	c-erbB-2 DNA Amplification[b]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[c]
Absence of estrogen receptors	<0.05	(253)[109] (141)[35] (109)[34] (86)[79] (50)[44] (47)[13]	(104)[35]	(350)[85c] (330)[17a] (185)[101]
	0.05–0.15	—	—	—
	>0.15	(157)[113] (122)[4] (103)[79] (95)[90] (64)[77] (61)[50] (58)[111] (53)[21] (51)[62] (41)[93]	(180)[69] (62)[65] (62)[35] (57)[50]	(290)[68] (172)[11] (51)[62c] (38)[13]
Absence of progesterone receptors	<0.05	(253)[109] (141)[35] (109)[34] (50)[44]	—	(350)[85c] (306)[17b]
	0.05–0.15	(86)[79] (49)[62]	—	—
	>0.15	(157)[113] (122)[4] (103)[79] (64)[77]	(180)[68] (103)[95] (62)[65] (56)[35]	(90)[11] (49)[62c]
Age (menopausal status)	<0.05	(younger: 86)[79]	—	(younger: 330)[17c] (older: 58)[52c]
	0.05–0.15	(230)[17] (176)[87] (157)[113] (122)[4] (116)[34] (103)[79] (95)[90] (64)[77] (58)[111] (56)[52] (53)[21] (49)[13] (41)[93] (15)[91]	(62)[65]	(350)[85c] (290)[98] (189)[92] (162)[11] (45)[92]
	>0.15			

[a] A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15

[b] Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.

[c] By Western blot method; all other protein studies used immunohistochemical methods.

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TABLE 5. CORRELATION OF c-erbB-2 ACTIVATION WITH OUTCOME IN PATIENTS WITH BREAST CARCINOMA

		Number of Patients				
P[a]	Type of c-erbB-2 Activation[b]	Total	With Metastasis to Axillary Lymph Nodes	No Metastasis	Statistical Analysis[c]	Reference
<0.05	DNA	176			M	87
<0.05	DNA	61			U	50
<0.05	DNA	57			U	65
<0.05	DNA	41			U	93
<0.05	mRNA	62			U	65
<0.05	Protein	102			M	101
<0.05	DNA		345		M	81
<0.05	DNA		120		U	17
<0.05	DNA		91		U	87
<0.05	DNA		86		M	79
<0.05	Protein-WB		350		M	85
<0.05	Protein		62	44	U	101
0.05–0.15	DNA	57			U	111
0.05–0.15	Protein	189			M	92
0.05–0.15	Protein		120		U	86
>0.15	DNA	130			U	113
>0.15	DNA	122			M	4
>0.15	DNA	50			U	44
>0.15	mRNA	57			U	50
>0.15	Protein	290			M	86
>0.15	Protein	195			U	11
>0.15	Protein	102			U	39
>0.15	Protein		137		U	17
>0.15	DNA			181	M	81
>0.15	DNA			159	U	17
>0.15	DNA			73	U	87
>0.15	Protein-WB			378	U	85
>0.15	Protein-WB			192	U	17
>0.15	Protein			141	U	86
>0.15	Protein			41	U	40

[a]The endpoints of these studies were tumor recurrence or decreased survival or both. Correlation between c-erbB-2 activation and a poorer patient outcome is statistically significant at <0.05, is of equivocal significance at 0.05 to 0.15, and is not significant at >0.15.
[b]Shown as variable measured. Letters "WB" indicate assay by Western blot; the other protein studies used immunohistochemical methods.
[c]M = multivariate statistical analysis; U = univariate statistical analysis.

TABLE 6. PERCENTAGE OF BREAST CARCINOMAS WITH METASTASIS COMPARED WITH PROGNOSTIC SIGNIFICANCE OF c-erbB-2 ACTIVATION

% of tumors with lymph node metastasis in each study

```
70 -    71 (DNA)[20]


                                    64(DNA)[111]          64 (mRNA)[50]
60 -    61 (DNA)[50]                                      61(DNA)[4]

        59 (DNA)[67]
        58(Protein)[101]                                  58 (DNA)[44]
                                                          57 (DNA)[113]
                                                          55(Protein)[39]

50 -                                                      48(Protein)[11]
                                                          46(Protein)[86]

                                    42(Protein)[92]
40 -

        P<0.05          0.05<P<0.15          P>0.15
```
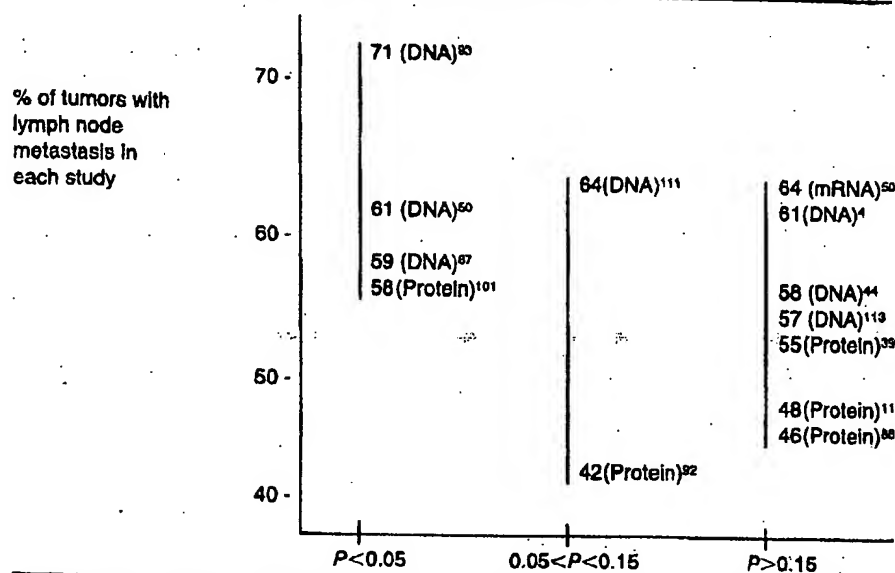
P for correlation of c-erbB-2 activation with patient outcome.

Each study's percentage of breast carcinomas with metastasis is compared with the correlation between c-erbB-2 activation and outcome. These data include only those studies that considered, as one group, all breast cancer patients, whether or not they had axillary metastasis. Superscripts are the references. In parentheses are the types of c-erbB-2 activation. *P* values are interpreted as in Table 3.

A second problem is that various types of breast carcinoma are grouped together in many survival studies. Because the current literature suggests that c-erbB-2 activation is infrequent in lobular carcinoma, studies that combine infiltrating ductal and lobular carcinomas may dilute the prognostic effect of c-erbB-2 activation in ductal tumors. In addition, most studies do not analyze inflammatory breast carcinoma separately. This condition frequently shows c-erbB-2 activation and has a worse prognosis than the usual mammary carcinoma, but it is an uncommon lesion.

A third potential problem is the paucity of studies that attempt to correlate c-erbB-2 activation with clinical outcome in subsets of breast carcinoma without metastasis. Two recent abstracts reported that in patients without lymph node metastasis who had various risk factors for recurrence (such as large tumor size and absence of estrogen receptors), c-erbB-2 overexpression predicted early recurrence.[23,67] In patients with ductal carcinoma in situ, one small study found no association between tumor recurrence and c-erbB-2 activation.[40]

A fourth problem is the lack of data regarding whether the prognosis correlates better with c-erbB-2 DNA amplification or with mRNA or protein overproduction. Most studies that find a correlation between c-erbB-2 activa-

tion and poor patient outcome measure c-erbB-2 DNA amplification (Table 5), and breast carcinoma patients with greater amplification of c-erbB-2 may have poorer survival.[79,61] Recent studies suggest that amplification has more prognostic power than overproduction,[17,34,35] but the clinical significance of c-erbB-2 overproduction without DNA amplification deserves further research.[17,52] Few studies have attempted to correlate patient outcome with c-erbB-2 mRNA overproduction, and many studies of c-erbB-2 protein overproduction use relatively less reliable methods such as immunohistochemical studies with polyclonal antibodies.

Comparison of c-erbB-2 Activation With Other Oncogenes in Breast Carcinoma

Other oncogenes that may have prognostic implications in human breast cancer are reviewed elsewhere.[71,106] This section will be restricted to a comparison between the clinical relevance of c-erbB-2 and these other oncogenes.

The c-myc gene is often activated in breast carcinomas, but c-myc activation generally has less prognostic importance than c-erbB-2 activation.[21,34,77,87,93] One study found a correlation between increased mRNAs of c-erbB-2 and c-myc, although other reports have not confirmed this.[34,106] Subsequent research, however, could demonstrate a subset of breast carcinomas in which c-myc has more prognostic importance than c-erbB-2.

The gene c-erbB-1 for the epidermal growth factor receptor (EGFR) is homologous with c-erbB-2 but is infrequently amplified in breast carcinomas.[79] Overproduction of EGFR, however, occurs more frequently than amplification and may correlate with a poor prognosis. In studies that have examined both c-erbB-2 and EGFR in the same tumor, c-erbB-2 has a stronger correlation with poor prognostic factors.[35,52] Studies have tended to show no correlation between amplification of c-erbB-2 and c-erbB-1 or overproduction of c-erbB-2 and EGFR, although at the molecular level EGFR mediates phosphorylation of c-erbB-2 protein.[51,52,61,88,109] Recent reviews describe EGFR in breast carcinoma.[43,100]

The genes c-erbA and ear-1 are homologous to the thyroid hormone receptor, and they are located adjacent to c-erbB-2 on chromosome 17. These genes are frequently coamplified with c-erbB-2 in breast carcinomas. The absence of c-erbA expression in breast carcinomas, however, is evidence against an important role for this gene in breast neoplasia.[90] Amplification of c-erbB-2 can occur without ear-1 amplification, and these tumors have a decreased survival that is similar to tumors with both c-erbB-2 and ear-1 amplification.[87] Consequently, c-erbB-2 amplification seems to be more important than amplification of c-erbA or ear-1.

Other genes also have been compared with c-erbB-2 activation in breast carcinomas. One study found a significant correlation between increased c-erbB-2 mRNA and increased mRNAs of fos, platelet-derived growth factor chain A, and Ki-ras.[106] Allelic deletion of c-Ha-ras may indicate a poorer prognosis in breast carcinoma,[21] but it has not been compared with c-erbB-2 activation. Some studies have suggested a correlation between advanced stage or recurrence of breast carcinoma and activation of any one of several oncogenes.[21,113]

ACTIVATION OF c-erbB-2 IN NON-MAMMARY TISSUES

Incidence of c-erbB-2 Activation in Non-Mammary Tissues

Table 7 summarizes the normal tissues in which c-erbB-2 expression has been detected, usually with immunohistochemical methods using polyclonal anti-

TABLE 7. PRESENCE OR ABSENCE OF c-erbB-2 mRNA OR c-erbB-2 PROTEIN IN NORMAL HUMAN TISSUES

Tissues With c-erbB-2 mRNA	Tissues Producing c-erbB-2 Protein[a]	Tissues Lacking c-erbB-2 mRNA	Tissues Lacking c-erbB-2 Protein
Skin[24]	Epidermis[56]		
	External root sheath[56]		
	Eccrine sweat gland[56]		
	Fetal oral mucosa[62]		Postnatal oral mucosa[62]
	Fetal esophagus[62]		Postnatal esophagus[62]
Stomach[24]	Stomach[22,62]		
	Fetal intestine[62a]		
Jejunum[24]	Small intestine[22,62]		
Colon[24]	Colon[22,62]		
Kidney[24]	Fetal kidney[62a]	Kidneys[104]	Glomerulus[62]
			Postnatal Bowman's capsule[62]
	Fetal proximal tubule[62]		Postnatal proximal tubule[62]
	Distal tubule[62]		
	Fetal collecting duct[62]		Postnatal collecting duct[62]
	Fetal renal pelvis[62]		Postnatal renal pelvis[62]
	Fetal ureter[62]		Postnatal fetal ureter[62]
Liver[24]	Hepatocytes[22]		Liver[62,95]
	Pancreatic acini[22]		
	Pancreatic ducts[22,62]		
	Endocrine cells of islets of Langerhans[22]		Pancreatic islets[62]
Lung[24]	Fetal trachea[62]		Postnatal trachea[62]
	Fetal bronchioles[62]		Postnatal bronchioles[62]
	Bronchioles[99]		
			Postnatal alveoli[62,99]
Fetal brain[24]			Postnatal brain[62]
	Fetal ganglion cells[62]		Postnatal ganglion cells[62]
Thyroid[1]			
Uterus[24]			
	Ovary[12]		
	Blood vessels[42]		Endothelium[62]
Placenta[24]			
			Adrenocortical cells[62]
			Postnatal thymus[62]
			Fibroblasts[62]
			Smooth muscle cells[62]
			Cardiac muscle cells[62]

[a]This protein study used Western blots; the rest used immunohistochemical methods.

bodies. Only a few studies have been performed, and some of these do not demonstrate convincing cell membrane reactivity in the published photographs. The interpretations in these studies, however, are listed, with the caveat that these findings should be confirmed by immunoprecipitation or Western or RNA blots. Production of c-erbB-2 has been identified in normal epithelium of the gastrointestinal tract and skin. Discrepancies regarding c-erbB-2 protein in other tissues could be due, at least in part, to differences in techniques.

The data on c-erbB-2 activation in various non-mammary neoplasms should be interpreted with caution, because only small numbers of tumors have been studied, usually by immunohistochemical methods using polyclonal antibodies. Studies using cell lines have been excluded, because cell culture can induce amplification and overexpression of other genes, although this has not been documented for c-erbB-2.

Activation of c-erbB-2 has been identified in 32 percent (64 of 203) of ovarian carcinomas in eight studies (Table 8). One abstract[45] stated that ovarian carcinomas contained significantly more c-erbB-2 protein than ovarian non-epithelial malignancies. Another report[81] showed that 12 percent of ovarian carcinomas had c-erbB-2 overproduction without amplification.

Activation of c-erbB-2 has been identified in 20 percent (40 of 198) of gastric adenocarcinomas in seven studies, including 33 percent (21 of 64) of

TABLE 8. c-erbB-2 ACTIVATION IN HUMAN GYNECOLOGIC TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Over-production	c-erbB-2 Protein Over-production
Ovary—carcinoma, not otherwise specified	31/120,[81] 1/11,[57] 0/5,[107] 0/5,[84] 0/3,[112] 0/2,[72] 0/1[110]	23/67[81]	23/73,[12] 36/72[81]
Ovary—serous (papillary) carcinoma	2/7,[110] 1/7,[112] 0/6[72]	—	—
Ovary—endometrioid carcinoma	0/3[110]	—	—
Ovary—mucinous carcinoma	1/2,[110] 0/1[72]	—	—
Ovary—clear cell carcinoma	0/2,[112] 0/1[72]	—	—
Ovary—mixed epithelial carcinoma	0/2[72]	—	—
Ovary—endometrioid borderline tumor	0/1[72]	—	—
Ovary—mucinous borderline tumor	0/3[72]	—	—
Ovary—serous cystadenoma	0/4[72]	—	—
Ovary—mucinous cystadenoma	0/2[72]	—	—
Ovary—sclerosing stromal tumor	0/1[72]	—	—
Ovary—fibrothecoma	0/1[72]	—	—
Uterus—endometrial adenocarcinoma	0/4,[84] 0/1[110]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

intestinal or tubular subtypes and 9 percent (4 of 47) of diffuse or signet ring cell subtypes (Table 9). Activation of c-erbB-2 has been detected in 2 percent (6 of 281) of colorectal carcinomas, although an additional immunohistochemical study detected c-erbB-2 protein in seven of eight tissues fixed in Bouin's solution. One study found greater immunohistochemical reactivity for c-erbB-2 protein in colonic adenomatous polyps than in the adjacent normal epithelium, using Bouin's fixative. Lesions with anaplastic features and progression to invasive carcinoma tended to show decreased immunohistochemical reactivity for c-erbB-2 protein.[22] Hepatocellular carcinomas (12 of 14 cases) and cholangiocarcinomas (46 of 63 cases) reacted with antibodies against c-erbB-2 in one study, but some of these "positive" cases showed only diffuse cytoplasmic staining, which

TABLE 9. c-erbB-2 ACTIVATION IN HUMAN GASTROINTESTINAL TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 Protein Over-production
Esophagus—squamous cell carcinoma	0/1[107]	0/1[61]
Stomach—carcinoma, poorly differentiated	0/22[108]	—
Stomach—adenocarcinoma	2/24,[94] 2/9,[107] 2/8,[111] 2/6,[57] 0/1[108]	4/27,[29] 3/10[61]
Stomach—carcinoma, intestinal or tubular type	5/10[108]	16/54[29]
Stomach—carcinoma, diffuse or signet ring cell type	0/2[108]	4/45[29]
Colorectum—carcinoma	2/49,[94] 1/45,[111] 1/45,[57] 1/45,[60] 0/40,[81] 0/32,[107] 0/3[82]	1/22,[58] 7/8[22b]
Colon—villous adenoma	0/1[60]	—
Colon—tubulovillous adenoma	0/5[60]	—
Colon—tubular adenoma	0/7[60]	19/19[22b]
Colon—hyperplastic polyp	0/1[60]	—
Intestine—leiomyosarcoma	—	0/1[61]
Hepatocellular carcinoma	0/12[111]	12/14,[95] 0/2[61]
Hepatoblastoma	0/1[57]	—
Cholangiocarcinoma	—	46/63[95]
Pancreas—adenocarcinoma	—	2/80,[41c] 0/2[61]
Pancreas—acinar carcinoma	—	0/1[41]
Pancreas—clear cell carcinoma	—	0/2[41]
Pancreas—large cell carcinoma	—	0/3[41]
Pancreas—signet ring carcinoma	—	0/1[41]
Pancreas—chronic inflammation	—	0/14[41c]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-erbB-2 mRNA.
[b]Tissues fixed in Bouin's solution.
[c]Only cases with distinct membrane staining are interpreted as showing c-erbB-2 overproduction.

TABLE 10. c-erbB-2 ACTIVATION IN HUMAN PULMONARY TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 Protein Overproduction
Non-small cell carcinoma	2/60,[75] 0/60[81]	1/84[59]
Epidermoid carcinoma	0/13,[52] 0/10,[57] 0/6[20]	3/5[99]
Adenocarcinoma	0/21,[52] 1/13,[20] 0/7,[111] 0/7,[57] 0/3[107]	4/12[99]
Large cell carcinoma	0/9,[52] 0/6[20]	—
Small cell carcinoma	—	0/26,[58] 0/3[99]
Carcinoid tumor	0/1[62]	0/3[99]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-erbB-2 mRNA.

does not indicate c-erbB-2 activation in breast neoplasms.[95] Also, some pancreatic carcinomas and chronic pancreatitis tissue had cytoplasmic immunohistochemical reactivity for c-erbB-2 protein, in addition to the rare case of pancreatic adenocarcinoma with distinct cell membrane staining.[41]

Tables 10 through 14 summarize the studies of c-erbB-2 activation in other neoplasms. The c-erbB-2 oncogene is not activated in most of these tumors. Activation of c-erbB-2 has been detected in 1 percent (4 of 299) of pulmonary non-small cell carcinomas in nine studies, although one additional report[99] found c-erbB-2 protein overproduction in 41 percent (7 of 17). Renal cell carcinoma had c-erbB-2 activation in 7 percent (2 of 30) in four studies. Overproduction of c-erbB-2 protein was described in one transitional cell carcinoma of the urinary bladder, a grade 2 papillary lesion.[58] Squamous cell carcinoma and basal cell carcinoma of the skin may contain c-erbB-2 protein, but it is not clear

TABLE 11. c-erbB-2 ACTIVATION IN HUMAN HEMATOLOGIC PROLIFERATIONS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction
Hematologic malignancies	0/23[111]	—	—
Malignant lymphoma	0/9,[57] 0/3[107]	0/1[1]	0/15[61]
Acute leukemia	0/14[57]	—	—
Acute lymphoblastic leukemia	0/1[107]	—	—
Acute myeloblastic leukemia	0/3[107]	—	—
Chronic leukemia	0/19[57]	—	—
Chronic lymphocytic leukemia	0/6[107]	—	—
Chronic myelogenous leukemia	0/8[107]	—	—
Myeloproliferative disorder	0/1[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

TABLE 12. c-erbB-2 ACTIVATION IN HUMAN TUMORS OF SOFT TISSUE AND BONE[a]

Tumor Type	c-erbB-2 DNA Amplification
Sarcoma	0/10,[111] 0/8[57]
Malignant fibrous histiocytoma	0/1[107]
Liposarcoma	0/3[107]
Pleomorphic sarcoma	0/1[107]
Rhabdomyosarcoma	0/1[107]
Osteogenic sarcoma	0/2,[107] 0/2[57]
Chondrosarcoma	0/1[107]
Ewing's sarcoma	0/1[57]
Schwannoma	0/1[57]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. No studies analyzed for c-erbB-2 mRNA or c-erbB-2 protein.

whether the protein level is increased over that of normal skin.[56] Thyroid carcinomas and adenomas can have low levels of increased c-erbB-2 mRNA. One abstract described low-level c-erbB-2 DNA amplification in one of ten salivary gland pleomorphic adenomas.[48]

Correlation of c-erbB-2 Activation With Patient Outcome

Very few studies have attempted to correlate c-erbB-2 activation in non-mammary tumors with outcome. Slamon et al[81] showed that c-erbB-2 amplification or overexpression in ovarian carcinomas correlates with decreased survival, especially when marked activation is present. However, they did not report the stage, histological grade, or histological subtype of these neoplasms. Another study of stages III and IV ovarian carcinomas found a correlation between decreased survival and c-erbB-2 protein overproduction, but not between survival and histological grade.[12] One abstract stated that c-erbB-2 protein overproduction in 10 of 16 pulmonary adenocarcinomas correlated with decreased disease-free interval.[70] Another abstract described a tendency for immunohisto-

TABLE 13. c-erbB-2 ACTIVATION IN HUMAN TUMORS OF THE URINARY TRACT[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Over-production	c-erbB-2 Protein Over-production
Kidney—renal cell carcinoma	1/5,[57] 1/4,[107] 0/5[94]	0/16[104]	—
Wilms' tumor	0/4[57]	—	—
Prostate—adenocarcinoma	—	—	0/23[58]
Urinary bladder—carcinoma	—	—	1/48[58]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

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TABLE 14. c-erbB-2 ACTIVATION IN MISCELLANEOUS HUMAN TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Over-production
Skin—malignant melanoma	—	—	0/10[58]
Skin, head and neck—squamous cell carcinoma	0/7[107]	—	—
Site not stated—squamous cell carcinoma	0/8,[57] 0/2[76]	—	—
Salivary gland—adenocarcinoma	1/1[76]	—	—
Parotid gland—adenoid cystic carcinoma	—	—	0/1[61]
Thyroid—anaplastic carcinoma	0/1[1]	0/1[1]	—
Thyroid—papillary carcinoma	0/5[1]	3(low levels)/5[1]	—
Thyroid—adenocarcinoma	0/1[84]	—	—
Thyroid—adenoma	0/2[1]	1(low levels)/2[1]	—
Neuroblastoma	0/35,[61] 0/9,[57] 0/1[76]	—	—
Meningioma	0/2[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

chemical reactivity for c-erbB-2 protein to correlate with higher grades of prostatic adenocarcinoma.[97] Additional prognostic studies of ovarian carcinomas and other neoplasms are needed.

SUMMARY

Activation of the c-erbB-2 oncogene can occur by amplification of c-erbB-2 DNA and by overproduction of c-erbB-2 mRNA and c-erbB-2 protein. Approximately 20 percent of breast carcinomas show evidence of c-erbB-2 activation, which correlates with a poor prognosis primarily in patients with metastasis to axillary lymph nodes. Studies that have attempted to correlate c-erbB-2 activation with other prognostic factors in breast carcinoma have reported conflicting conclusions. The pathologic and clinical significance of c-erbB-2 activation in other neoplasms is unclear and should be assessed by additional studies.

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Research

Prostate stem cell antigen (PSCA) expression in human prostate cancer tissues and its potential role in prostate carcinogenesis and progression of prostate cancer

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Abstract

Background: Prostate stem cell antigen (PSCA) is a recently defined homologue of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. The purpose of the present study was to examine the expression status of PSCA protein and mRNA in clinical specimens of human prostate cancer (Pca) and to validate it as a potential molecular target for diagnosis and treatment of Pca.

Materials and Methods: Immunohistochemical (IHC) and *in situ* hybridization (ISH) analyses of PSCA expression were simultaneously performed on paraffin-embedded sections from 20 benign prostatic hyperplasia (BPH), 20 prostatic intraepithelial neoplasm (PIN) and 48 prostate cancer (Pca) tissues, including 9 androgen-independent prostate cancers. The level of PSCA expression was semiquantitatively scored by assessing both the percentage and intensity of PSCA-positive staining cells in the specimens. Then compared PSCA expression between BPH, PIN and Pca tissues and analysed the correlations of PSCA expression level with pathological grade, clinical stage and progression to androgen-independence in Pca.

Results: In BPH and low grade PIN, PSCA protein and mRNA staining were weak or negative and less intense and uniform than that seen in HGPIN and Pca. There were moderate to strong PSCA protein and mRNA expression in 8 of 11 (72.7%) HGPIN and in 40 of 48 (83.4%) Pca specimens examined by IHC and ISH analyses, with statistical significance compared with BPH (20%) and low grade PIN (22.2%) samples ($p < 0.05$, respectively). The expression level of PSCA increased with high Gleason grade, advanced stage and progression to androgen-independence ($p < 0.05$, respectively). In addition, IHC and ISH staining showed a high degree of correlation between PSCA protein and mRNA overexpression.

Conclusions: Our data demonstrate that PSCA as a new cell surface marker is overexpressed by a majority of human Pca. PSCA expression correlates positively with adverse tumor characteristics, such as increasing pathological grade (poor cell differentiation), worsening clinical stage and androgen-independence, and speculatively with prostate carcinogenesis. PSCA protein overexpression results from upregulated transcription of PSCA mRNA. PSCA may have prognostic utility and may be a promising molecular target for diagnosis and treatment of Pca.

Introduction

Prostate cancer (Pca) is the second leading cause of cancer-related death in American men and is becoming a common cancer increasing in China. Despite recently great progress in the diagnosis and management of localized disease, there continues to be a need for new diagnostic markers that can accurately discriminate between indolent and aggressive variants of Pca. There also continues to be a need for the identification and characterization of potential new therapeutic targets on Pca cells. Current diagnostic and therapeutic modalities for recurrent and metastatic Pca have been limited by a lack of specific target antigens of Pca.

Although a number of prostate-specific genes have been identified (i.e. prostate specific antigen, prostatic acid phosphatase, glandular kallikrein 2), the majority of these are secreted proteins not ideally suited for many immunological strategies. So, the identification of new cell surface antigens is critical to the development of new diagnostic and therapeutic approaches to the management of Pca.

Reiter RE et al [1] reported the identification of prostate stem cell antigen (PSCA), a cell surface antigen that is predominantly prostate specific. The PSCA gene encodes a 123 amino acid glycoprotein, with 30% homology to stem cell antigen 2 (Sca 2). Like Sca-2, PSCA also belongs to a member of the Thy-1/Ly-6 family and is anchored by a glycosylphosphatidylinositol (GPI) linkage. mRNA *in situ* hybridization (ISH) localized PSCA expression in normal prostate to the basal cell epithelium, the putative stem cell compartment of prostatic epithelium, suggesting that PSCA may be a marker of prostate stem/progenitor cells.

In order to examine the status of PSCA protein and mRNA expression in human Pca and validate it as a potential diagnostic and therapeutic target for Pca, we used immunohistochemistry (IHC) and *in situ* hybridization (ISH) simultaneously, and conducted PSCA protein and mRNA expression analyses in paraffin-embedded tissue specimens of benign prostatic hyperplasia (BPH, n = 20), prostate intraepithelial neoplasm (PIN, n = 20) and prostate cancer (Pca, n = 48). Furthermore, we evaluated the possible correlation of PSCA expression level with Pca tumorigenesis, grade, stage and progression to androgen-independence.

Materials and methods

Tissue samples

All of the clinical tissue specimens studied herein were obtained from 80 patients of 57–84 years old by prostatectomy, transurethral resection of prostate (TURP) or biopsies. The patients were classified as 20 cases of BPH, 20 cases of PIN, 40 cases of primary Pca, including 9 patients

with recurrent Pca and a history of androgen ablation therapy (orchiectomy and/or hormonal therapy), who were referred to as androgen-independent prostate cancers. Eight specimens were harvested from these androgen-independent Pca patients prior to androgen ablation treatment. Each tissue sample was cut into two parts, one was fixed in 10% formalin for IHC and the other treated with 4% paraformaldehyde/0.1 M PBS PH 7.4 in 0.1% DEPC for 1 h for ISH analysis, and then embedded in paraffin. All paraffin blocks examined were then cut into 5 µm sections and mounted on the glass slides specific for IHC and ISH respectively in the usual fashion. H&E-stained section of each Pca was evaluated and assigned a Gleason score by the experienced urological pathologist at our institution based on the criteria of Gleason score [2]. The Gleason sums are summarized in Table 1. Clinical staging was performed according to Jewett-whitmore-prout staging system, as shown in Table 2. In the category of PIN, we graded the specimens into two groups, i.e. low grade PIN (grade I – II) and high grade PIN (HGPIN, grade III) on the basis of literatures [3,4].

Immunohistochemical (IHC) analysis

Briefly, tissue sections were deparaffinized, dehydrated, and subjected to microwaving in 10 mmol/L citrate buffer, PH 6.0 (Boshide, Wuhan, China) in a 900 W oven for 5 min to induce epitope retrieval. Slides were allowed to cool at room temperature for 30 min. A primary mouse antibody specific to human PSCA (Boshide, Wuhan, China) with a 1:100 dilution was applied to incubate with the slides at room temperature for 2 h. Labeling was detected by sequentially adding biotinylated secondary antibodies and strepavidin-peroxidase, and localized using 3,3'-diaminobenzidine reaction. Sections were then counterstained with hematoxylin. Substitution of the primary antibody with phosphate-buffered-saline (PBS) served as a negative-staining control.

mRNA in situ hybridization (ISH)

Five-µm-thick tissue sections were deparaffinized and dehydrated, then digested in pepsin solution (4 mg/ml in 3% citric acid) for 20 min at 37.5°C, and further processed for ISH. Digoxigenin-labeled sense and antisense human PSCA RNA probes (obtained from Boshide, Wuhan, China) were hybridized to the sections at 48°C overnight. The posthybridization wash with a high stringency was performed sequentially at 37°C in 2 × standard saline citrate (SSC) for 10 min, in 0.5 × SSC for 15 min and in 0.2 × SSC for 30 min. The slides were then incubated to biotinylated mouse anti-digoxigenin antibody at 37.5°C for 1 h followed by washing in 1 × PBS for 20 min at room temperature, and then to strepavidin-peroxidase at 37.5°C for 20 min followed by washing in 1 × PBS for 15 min at room temperature. Subsequently, the slides were developed with diaminobenzidine and then coun-

Table 1: Correlation of PSCA expression with Gleason score

Gleason score	Intensity × frequency	
	0–6 (%)	9 (%)
2–4	5 (83)	1 (17)
5–7	19 (79)	5 (21)
8–10	5 (28)	13 (72)

Table 2: Correlation of PSCA expression with clinical stage

Tumor stage	Intensity × frequency	
	0–6 (%)	9 (%)
≤B	27 (67.5)	13 (32.5)
≥C	2 (25)	6 (75)

terstained with hematoxylin to localize the hybridization signals. Sections hybridized with the sense control probes routinely did not show any specific hybridization signal above background. All slides were hybridized with PBS to substitute for the probes as a negative control.

Scoring methods
To determine the correlation between the results of PSCA immunostaining and mRNA *in situ* hybridization, the same scoring manners are taken in the present study for PSCA protein staining by IHC and PSCA mRNA staining by ISH. Each slide was read and scored by two independently experienced urological pathologists using Olympus BX-41 light microscopes. The evaluation was done in a blinded fashion. For each section, five areas of similar grade were analyzed semiquantitatively for the fraction of cells staining. Fifty percent of specimens were randomly chosen and rescored to determine the degree of interobserver and intraobserver concordance. There was greater than 95% intra- and interobserver agreement.

The intensity of PSCA expression evaluated microscopically was graded on a scale of 0 to 3+ with 3 being the highest expression observed (0, no staining; 1+, mildly intense; 2+, moderately intense; 3+, severely intense). The staining density was quantified as the percentage of cells staining positive for PSCA with the primary antibody or hybridization probe, as follows: 0 = no staining; 1 = positive staining in <25% of the sample; 2 = positive staining in 25%–50% of the sample; 3 = positive staining in >50%

of the sample. Intensity score (0 to 3+) was multiplied by the density score (0–3) to give an overall score of 0–9 [1,5]. In this way, we were able to differentiate specimens that may have had focal areas of increased staining from those that had diffuse areas of increased staining [6]. The overall score for each specimen was then categorically assigned to one of the following groups: 0 score, negative expression; 1–2 scores, weak expression; 3–6 scores, moderate expression; 9 score, strong expression.

Statistical analysis
Intensity and density of PSCA protein and mRNA expression in BPH, PIN and Pca tissues were compared using the Chi-square and Student's *t*-test. Univariate associations between PSCA expression and Gleason score, clinical stage and progression to androgen-independence were calculated using Fisher's Exact Test. For all analyses, p < 0.05 was considered statistically significant.

Results
PSCA expression in BPH
In general, PSCA protein and mRNA were expressed weakly in individual samples of BPH. Some areas of prostate expressed weak levels (composite score 1–2), whereas other areas were completely negative (composite score 0). Four cases (20%) of BPH had moderate expression of PSCA protein and mRNA (composite score 4–6) by IHC and ISH. In 2/20 (10%) BPH specimens, PSCA mRNA expression was moderate (composite score 3–6), but PSCA protein expression was weak (composite score

2) in one and negative (composite score 0) in the other. PSCA expression was localized to the basal and secretory epithelial cells, and prostatic stroma was almost negative staining for PSCA protein and mRNA in all cases examined.

PSCA expression in PIN

In this study, we detected weak or negative expression of PSCA protein and mRNA (≤ 2 scores) in 7 of 9 (77.8%) low grade PIN and in 2 of 11 (18.2%) HGPIN, and moderate expression (3–6 scores) in the rest 2 low grade PIN and 5 of 11 (45.5%) HGPIN. One HGPIN with moderate PSCA mRNA expression (6 score) was found weak staining for PSCA protein (2 score) by IHC. Strong PSCA protein and mRNA expression (9 score) were detected in the remaining 3 of 11 (27.3%) HGPIN. There was a statistically significant difference of PSCA protein and mRNA expression levels observed between HGPIN and BPH ($p < 0.05$), but no statistical difference reached between low grade PIN and BPH ($p > 0.05$).

PSCA expression in Pca

In order to determine if PSCA protein and mRNA can be detected in prostate cancers and if PSCA expression levels are increased in malignant compared with benign glands, Forty-eight paraffin-embedded Pca specimens were analysed by IHC and ISH. It was shown that 19 of 48 (39.6%) Pca samples stained very strongly for PSCA protein and mRNA with a score of 9 and another 21 (43.8%) specimens displayed moderate staining with scores of 4–6 (Figure 1). In addition, 4 specimens with moderate to strong PSCA mRNA expression (scores of 4–9) had weak protein staining (a score of 2) by IHC analyses. Overall, Pca expressed a significantly higher level of PSCA protein and mRNA than any other specimen category in this study ($p < 0.05$, compared with BPH and PIN respectively). The result demonstrates that PSCA protein and mRNA are overexpressed by a majority of human Pca.

Correlation of PSCA expression with Gleason score in Pca

Using the semi-quantitative scoring method as described in Materials and Methods, we compared the expression level of PSCA protein and mRNA with Gleason grade of Pca, as shown in Table 1. Prostate adenocarcinomas were graded by Gleason score as 2–4 scores = well-differentiation, 5–7 scores = moderate-differentiation and 8–10 scores = poor-differentiation [7]. Seventy-two percent of Gleason scores 8–10 prostate cancers had very strong staining of PSCA compared to 21% with Gleason scores 5–7 and 17% with 2–4 respectively, demonstrating that poorly differentiated Pca had significantly stronger expression of PSCA protein and mRNA than moderately and well differentiated tumors ($p < 0.05$). As depicted in Figure 1, IHC and ISH analyses showed that PSCA protein and mRNA expression in several cases of poorly differen-

tiated Pca were particularly prominent, with more intense and uniform staining. The results indicate that PSCA expression increases significantly with higher tumor grade in human Pca.

Correlation of PSCA expression with clinical stage in Pca

With regards to PSCA expression in every stage of Pca, we showed the results in Table 2. Seventy-five percent of locally advanced and node positive cancers (i.e. C-D stages) expressed statistically high levels of PSCA versus 32.5% that were organ confined (i.e. A-B stages) ($p < 0.05$). The data demonstrate that PSCA expression increases significantly with advanced tumor stage in human Pca.

Correlation of PSCA expression with androgen-independent progression of Pca

All 9 specimens of androgen-independent prostate cancers stained positive for PSCA protein and mRNA. Eight specimens were obtained from patients managed prior to androgen ablation therapy. Seven of eight (87.5%) of these androgen-independent prostate cancers were in the strongest staining category (score = 9), compared with three out of eight (37.5%) of patients with androgen-dependent cancers ($p < 0.05$). The results demonstrate that PSCA expression increases significantly with progression to androgen-independence of human Pca.

It is evident from the results above that within a majority of human prostate cancers the level of PSCA protein and mRNA expression correlates significantly with increasing grade, worsening stage and progression to androgen-independence.

Correlation of PSCA immunostaining and mRNA in situ hybridization

In all 88 specimens surveyed herein, we compared the results of PSCA IHC staining with mRNA ISH analysis. Positive staining areas and its intensity and density scores evaluated by IHC were identical to those seen by ISH in 79 of 88 (89.8%) specimens (18/20 BPH, 19/20 PIN and 42/48 Pca respectively). Importantly, 27/27 samples with PSCA mRNA composite scores of 0–2, 32/36 samples with scores of 3–6 and 22/24 samples with a score of 9 also had PSCA protein expression scores of 0–2, 3–6 and 9 respectively. However, in 5 samples with PSCA mRNA overall scores of 3–6 and in 2 with scores of 9 there were less or negative PSCA protein expression (i.e. scores of 0–4), suggesting that this may reflect posttranscriptional modification of PSCA or that the epitopes recognized by PSCA mAb may be obscured in some cancers. The data demonstrate that the results of PSCA immunostaining were consistent with those of mRNA ISH analysis, showing a high degree of correlation between PSCA protein and mRNA expression.

Figure 1
Representatives of PSCA IHC and ISH staining in Pca (A. IHC staining, B. ISH staining, ×200 magnification). A_1, B_1: negative control of IHC and ISH. PBS replacing the primary antibody (A_1) and hybridization with a sense PSCA probe (B_1) showed no background staining. A_2, B_2: a moderately differentiated Pca (Gleason score = 3+3 = 6) with moderate staining (composite score = 6) in all malignant cells; A_2: IHC shows not only cell surface but also apparent cytoplasmic staining of PSCA protein. A_3, B_3: a poorly differentiated Pca (Gleason score = 4+4 = 8) with very strong staining (composite score = 9) in all malignant cells.

Discussion

PSCA is homologous to a group of cell surface proteins that mark the earliest phase of hematopoietic development. PSCA mRNA expression is prostate-specific in normal male tissues and is highly up-regulated in both androgen-dependent and-independent Pca xenografts (LAPC-4 tumors). We hypothesize that PSCA may play a role in Pca tumorigenesis and progression, and may serve as a target for Pca diagnosis and treatment. In this study, IHC and ISH showed that in general there were weak or absent PSCA protein and mRNA expression in BPH and low grade PIN tissues. However, PSCA protein and mRNA are widely expressed in HGPIN, the putative precursor of invasive Pca, suggesting that up-regulation of PSCA is an early event in prostate carcinogenesis. Recently, Reiter RE et al [1], using ISH analysis, reported that 97 of 118 (82%) HGPIN specimens stained strongly positive for PSCA mRNA. A very similar finding was seen on mouse PSCA (mPSCA) expression in mouse HGPIN tissues by Tran C. P et al [8]. These data suggest that PSCA may be a new marker associated with transformation of prostate cells and tumorigenesis.

We observed that PSCA protein and mRNA are highly expressed in a large percentage of human prostate cancers, including advanced, poorly differentiated, androgen-independent and metastatic cases. Fluorescence-activated cell sorting and confocal/ immunofluorescent studies demonstrated cell surface expression of PSCA protein in Pca cells [9]. Our IHC expression analysis of PSCA shows not only cell surface but also apparent cytoplasmic staining of PSCA protein in Pca specimens (Figure 1). One possible explanation for this is that anti-PSCA antibody can recognize PSCA peptide precursors that reside in the cytoplasm. Also, it is possible that the positive staining that appears in the cytoplasm is actually from the overlying cell membrane [5]. These data seem to indicate that PSCA is a novel cell surface marker for human Pca.

Our results show that elevated level of PSCA expression correlates with high grade (i.e. poor differentiation), increased tumor stage and progression to androgen-independence of Pca. These findings support the original IHC analyses by Gu Z et al [9], who reported that PSCA protein expressed in 94% of primary Pca and the intensity of PSCA protein expression increased with tumor grade, stage and progression to androgen-independence. Our results also collaborate the recent work of Han KR et al [10], in which the significant association between high PSCA expression and adverse prognostic features such as high Gleason score, seminal vesicle invasion and capsular involvement in Pca was found. It is suggested that PSCA overexpression may be an adverse predictor for recurrence, clinical progression or survival of Pca. Hara H et al [11] used RT-PCR detection of PSA, PSMA and PSCA in 1

ml of peripheral blood to evaluate Pca patients with poor prognosis. The results showed that among 58 PCa patients, each PCR indicated the prognostic value in the hierarchy of PSCA>PSA>PSMA RT-PCR, and extraprostatic cases with positive PSCA PCR indicated lower disease-progression-free survival than those with negative PSCA PCR, demonstrating that PSCA can be used as a prognostic factor. Dubey P et al [12] reported that elevated numbers of PSCA + cells correlate positively with the onset and development of prostate carcinoma over a long time span in the prostates of the TRAMP and PTEN +/- models compared with its normal prostates. Taken together with our present findings, in which PSCA is overexpressed from HGPIN to almost frank carcinoma, it is reasonable and possible to use increased PSCA expression level or increased numbers of PSCA-positive cells in the prostate samples as a prognostic marker to predict the potential onset of this cancer. These data raise the possibility that PSCA may have diagnostic utility or clinical prognostic value in human Pca.

The cause of PSCA overexpression in Pca is not known. One possible mechanism is that it may result from PSCA gene amplification. In humans, PSCA is located on chromosome 8q24.2 [1], which is often amplified in metastatic and recurrent Pca and considered to indicate a poor prognosis [13-15]. Interestingly, PSCA is in close proximity to the c-myc oncogene, which is amplified in >20% of recurrent and metastatic prostate cancers [16,17]. Reiter RE et al [18] reported that PSCA and MYC gene copy numbers were co-amplified in 25% of tumors (five out of twenty), demonstrating that PSCA overexpression is associated with PSCA and MYC coamplification in Pca. Gu Z et al [9] recently reporeted that in 102 specimens available to compare the results of PSCA immunostaining with their previous mRNA ISH analysis, 92 (90.2%) had identically positive areas of PSCA protein and mRNA expression. Taken together with our findings, in which we detected moderate to strong expression of PSCA protein and mRNA in 34 of 40 (85%) Pca specimens examined simultaneously by IHC and ISH analyses, it is demonstrated that PSCA protein and mRNA overexpressed in human Pca, and that the increased protein level of PSCA was resulted from the upregulated transcription of its mRNA.

At present, the regulation mechanisms of human PSCA expression and its biological function are yet to be elucidated. PSCA expression may be regulated by multiple factors [18]. Watabe T et al [19] reported that transcriptional control is a major component regulating PSCA expression levels. In addition, induction of PSCA expression may be regulated or mediated through cell-cell contact and protein kinase C (PKC) [20]. Homologues of PSCA have diverse activities, and have themselves been involved in

carcinogenesis. Signalling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes [21]. Thy-1 is involved in T cell activation and transducts signals through src-like tyrosine kinases [22]. Ly-6 genes have been implicated both in tumorigenesis and in cell-cell adhesion [23-25]. Cell-cell or cell-matrix interaction is critical for local tumor growth and spread to distal sites. From its restricted expression in basal cells of normal prostate and its homology to SCA-2, PSCA may play a role in stem/progenitor cell function, such as self-renewal (i.e. anti-apoptosis) and/or proliferation [1]. Taken together with the results in the present study, we speculate that PSCA may play a role in tumorigenesis and clinical progression of Pca through affecting cell transformation and proliferation. From our results, it is also suggested that PSCA as a new cell surface antigen may have a number of potential uses in the diagnosis, therapy and clinical prognosis of human Pca. PSCA overexpression in prostate biopsies could be used to identify patients at high risk to develop recurrent or metastatic disease, and to discriminate cancers from normal glands in prostatectomy samples. Similarly, the detection of PSCA-overexpressing cells in bone marrow or peripheral blood may identify and predict metastatic progression better than current assays, which identify only PSA-positive or PSMA-positive prostate cells.

In summary, we have shown in this study that PSCA protein and mRNA are maintained in expression from HGPIN through all stages of Pca in a majority of cases, which may be associated with prostate carcinogenesis and correlate positively with high tumor grade (poor cell differentiation), advanced stage and androgen-independent progression. PSCA protein overexpression is due to the upregulation of its mRNA transcription. The results suggest that PSCA may be a promising molecular marker for the clinical prognosis of human Pca and a valuable target for diagnosis and therapy of this tumor.

Competing interests
None declared.

References

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RNA Ladder

Molecular Weight Markers

④

#N0362S   25 µg $55

Bases

9,000 —
7,000 —
5,000 —
3,000 —
2,000 —
1,000 —
500 —

1 µg of RNA Ladder was heated at 60°C for 3 minutes in 1X RNA Ladder Sample Buffer and visualized by ethidium bromide staining (1.0% TBE agarose gel).
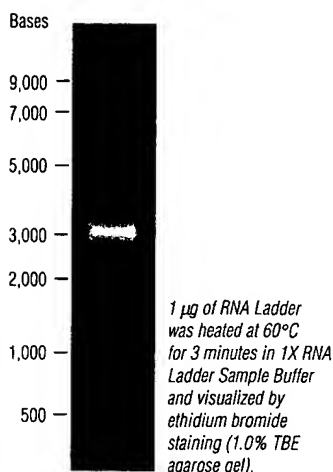
Description: The RNA Ladder is a set of 7 RNA molecules produced by *in vitro* transcription of a mixture of 7 linear DNA templates. The ladder sizes are: 9000, 7000, 5000, 3000, 2000, 1000 and 500 bases. The 3000 base fragment is at double intensity to serve as a reference band. This ladder is suitable for use as an RNA size standard on denaturing or native agarose gels.

Reagents Supplied with Ladders:
2X RNA Ladder Sample Buffer (for use with native agarose gels)

2X RNA Ladder Sample Buffer:
2X TBE (pH 8.3), 13% ficoll (w/v), 0.01% bromophenol blue and 7 M urea.

Concentration: 500 µg/ml.

Storage Conditions: 20 mM KOAc (pH 4.5). Store at –70°C. For short term storage (< 1 week), ladder can be stored at –20°C.

Notes on Use:
To avoid ribonuclease contamination: wear gloves, use RNase-free water for gels and buffers, wash equipment with detergent and rinse thoroughly with RNase-free water.

It is best to use freshly poured gels that are as thin as possible (i.e, 2–10 mm). Excessively long run times or

high voltage can cause degradation of the bands on the gel. We recommend 4–8 volts/cm and running the bromophenol blue approximately 5 cm into the gel for good resolution.

Adding ethidium bromide to agarose gels and running buffer at a final concentration of 0.5 µg/ml will effectively stain the bands during electrophoresis.

Denaturing vs. Native Agarose Gels:
It is common practice to electrophorese RNA on a fully denaturing agarose gel, such as one containing formaldehyde (1). However, in many cases it is possible to run RNA on a native agarose gel and obtain suitable results. In fact, it has been demonstrated that treatment of RNA samples in a denaturing buffer maintains the RNA molecules in a denatured state, during electrophoresis, for at least 3 hours (2,3). The use of native agarose gels eliminates problems associated with toxic chemicals and the difficulties encountered when staining and blotting formaldehyde gels.

References:
(1) Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 7.43–7.45). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
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(3) Sandra Cook and Christina Marchetti, unpublished observations.

2-Log DNA Ladder (0.1–10.0 kb)   New

#N3200S   100 µg $55
#N3200L   500 µg $220

DNA Mass (ng)	Kilobases
40	10.0 —
40	8.0 —
48	6.0 —
40	5.0 —
32	4.0 —
120	**3.0 —**
40	2.0 —
57	1.5 —
45	1.2 —
122	**1.0 —**
34	0.9 —
31	0.8 —
27	0.7 —
23	0.6 —
124	**0.5 —**
49	0.4 —
37	0.3 —
32	0.2 —
61	0.1 —

1.0 µg of 2-Log DNA Ladder visualized by ethidium bromide staining on a 1.0% TBE agarose gel.

Description: A number of proprietary plasmids are digested to completion with appropriate restriction enzymes to yield 19 bands suitable for use as molecular weight standards for agarose gel electrophoresis. This digested DNA includes fragments ranging from 100 bp to 10 kb. The 0.5, 1.0 and 3.0 kb bands have increased intensity to serve as reference points.

Preparation: Double-stranded DNA is digested to completion with the appropriate restriction enzymes, phenol extracted and equilibrated to 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Concentration: 1,000 µg/ml.

Storage Conditions: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. For long term storage, store at –20°C. 2-Log DNA Ladder is stable for at least 3 months at 4°C.

Note: All fragments have 4-base, 5´ overhangs that can be end labeled using T4 Polynucleotide Kinase (NEB #M0201) or filled-in using DNA Polymerase I, Klenow Fragment (NEB #M0210) (1). Use α-[^{32}P] dATP or α-[^{32}P] dTTP for the fill-in reaction.

Usage Recommendation: We recommend loading 1 µg of the 2-Log DNA Ladder diluted in sample buffer. This ladder was not designed for precise quantification of DNA mass but can be used for approximating the mass of DNA in comparably intense samples of similar size.

The approximate mass of DNA in each of the bands in our 2-Log DNA Ladder is as follows (assuming a 1 µg loading):

Fragment	Base Pairs	DNA Mass
1	10,002	40 ng
2	8,001	40 ng
3	6,001	48 ng
4	5,001	40 ng
5	4,001	32 ng
6	**3,001**	**120 ng**
7	2,017	40 ng
8	1,517	57 ng
9	1,200	45 ng
10	**1,000**	**122 ng**
11	900	34 ng
12	800	31 ng
13	700	27 ng
14	600	23 ng
15a	**517**	
15b	**500**	124 ng
16	400	49 ng
17	300	37 ng
18	200	32 ng
19	100	61 ng

Reference:
(1) Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 10.51–10.67). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

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Tue Feb 12 09:52:12 2002 [BLASTP 2.2.1 [Jul-12-2001], NCBI]
/home/ruby/va/Molbio/carpenda/tempids/p1.DNA45409 (234 aa)
        /home/ruby/va/Molbio/carpenda/tempids/p1.DNA45409


Sequences producing High-scoring Segment Pairs:          Score Match Pct E-val
   1 P_AAB87532  Human PRO1864 - Homo sapiens.            1195   234 100 e-130
   2 P_AAM41716  Human polypeptide SEQ ID NO 6647 - Homo sa 1195 234 100 e-130
   3 P_AAM41715  Human polypeptide SEQ ID NO 6646 - Homo sa 1195 234 100 e-130
   4 P_AAM39929  Human polypeptide SEQ ID NO 3074 - Homo sa 1195 234 100 e-130
   5 P_AAU29056  Human PRO polypeptide sequence #33 - Homo  1195 234 100 e-130
   6 AAH05959.1  Unknown (protein for MGC:14607) - Homo sap 1195 234 100 e-130
   7 AAH03074.1  Similar to steroidogenic acute regulatory 1195 234 100 e-130
   8 AAD15552.1  H_NH1021A08.1 - Homo sapiens             1195   234 100 e-130
   9 NP_114405.1 hypothetical protein MGC3251 - Homo sapien 1195 234 100 e-130
  10 P_AAM39930  Human polypeptide SEQ ID NO 3075 - Homo sa 1087 216  92 e-117

Dayhoff Protein Database (Rel 78, Mar 2004)


P_AAB87532  Human PRO1864 - Homo sapiens.
Length: 234 aa
Accession: P_AAB87532;
Species: Homo sapiens.
Keywords: Human; PRO protein; mapping; patent; GENESEQ patentdb.
Patent number: WO200116318-A2.
Publication date: 08-MAR-2001.
Filing date: 24-AUG-2000; 2000WO-US023328.
Priority: 01-SEP-1999; 99WO-US020111. 15-SEP-1999; 99WO-US021090.
        07-DEC-1999; 99US-0169495P. 09-DEC-1999; 99US-0170262P.
        11-JAN-2000; 2000US-0175481P. 18-FEB-2000; 2000WO-US004341.
        18-FEB-2000; 2000WO-US004342. 22-FEB-2000; 2000WO-US004414.
        01-MAR-2000; 2000WO-US005601. 03-MAR-2000; 2000US-0187202P.
        21-MAR-2000; 2000US-0191007P. 30-MAR-2000; 2000WO-US008439.
        25-APR-2000; 2000US-0199397P. 22-MAY-2000; 2000WO-US014042.
        05-JUN-2000; 2000US-0209832P.
Assignee: (GETH ) GENENTECH INC.
Inventors: Eaton DL, Filvaroff E, Gerritsen ME, Goddard A, Godowski PJ;
        Grimaldi CJ, Gurney AL, Watanabe CK, Wood WI;
Cross reference: WPI; 2001-183260/18. N-PSDB; AAF92064.
Title: Eighty four nucleic acids encoding PRO polypeptides, useful in
        molecular biology, including use as hybridization probes, and in
        chromosome and gene mapping.
Patent format: Claim 12; Fig 14; 278pp; English.
Comment: The present sequence is a human PRO polypeptide (secreted and
        transmembrane). The PRO protein, and PRO agonists, PRO antagonists
        or anti-PRO antibodies are useful for preparation of a medicament
        useful in the treatment of a condition which is responsive to the
        PRO protein, agonists, antagonists or anti-PRO antibodies. The PRO
        protein may also be employed as molecular weight markers for
        protein electrophoresis. The PRO coding sequence has applications
        in molecular biology, including use as hybridisation probes, and in
        chromosome and gene mapping
Database: GENESEQ patent database (v200420, 23-SEP-2004).


P_AAM41716  Human polypeptide SEQ ID NO 6647 - Homo sapiens.
Length: 238 aa
Accession: P_AAM41716;
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Species: Homo sapiens.
Keywords: Human; nootropic; immunosuppressant; cytostatic; gene therapy;
     cancer; peripheral nervous system; neuropathy; central nervous
     system; CNS; Alzheimer's; Parkinson's disease; Huntington's
     disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
     Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
     arthritis; inflammation; leukaemia; patent; GENESEQ patentdb.
Patent number: WO200153312-A1.
Publication date: 26-JUL-2001.
Filing date: 26-DEC-2000; 2000WO-US034263.
Priority: 23-DEC-1999; 99US-00471275. 21-JAN-2000; 2000US-00488725.
     25-APR-2000; 2000US-00552317. 20-JUN-2000; 2000US-00598042.
     19-JUL-2000; 2000US-00620312. 03-AUG-2000; 2000US-00653450.
     14-SEP-2000; 2000US-00662191. 19-OCT-2000; 2000US-00693036.
     29-NOV-2000; 2000US-00727344.
Assignee: (HYSE-) HYSEQ INC.
Inventors: Tang YT, Liu C, Asundi V, Chen R, Ma Y, Qian XB, Ren F, Wang D;
     Wang J, Wang Z, Wehrman T, Xu C, Xue AJ, Yang Y, Zhang J, Zhao QA;
     Zhou P, Goodrich R, Drmanac RT;
Cross reference: WPI; 2001-442253/47. N-PSDB; AAI60872.
Title: Novel nucleic acids and polypeptides, useful for treating disorders
     such as central nervous system injuries.
Patent format: Example 2; SEQ ID NO 6647; 10078pp; English.
Comment: The invention relates to human nucleic acids (AAI57798-AAI61369)
     and the encoded polypeptides (AAM38642-AAM42213) with nootropic,
     immunosuppressant and cytostatic activity. The polynucleotides are
     useful in gene therapy. A composition containing a polypeptide or
     polynucleotide of the invention may be used to treat diseases of
     the peripheral nervous system, such as peripheral nervous injuries,
     peripheral neuropathy and localised neuropathies and central
     nervous system diseases, such as Alzheimer's, Parkinson's disease,
     Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager
     Syndrome. Other uses include the utilisation of the activities such
     as: Immune system suppression, Activin/inhibin activity,
     chemotactic/chemokinetic activity, haemostatic and thrombolytic
     activity, cancer diagnosis and therapy, drug screening, assays for
     receptor activity, arthritis and inflammation, leukaemias and C.N.S
     disorders. Note: The sequence data for this patent did not form
     part of the printed specification
Database: GENESEQ patent database (v200420, 23-SEP-2004).

P_AAM41715 Human polypeptide SEQ ID NO 6646 - Homo sapiens.
Length: 238 aa
Accession: P_AAM41715;
Species: Homo sapiens.
Keywords: Human; nootropic; immunosuppressant; cytostatic; gene therapy;
     cancer; peripheral nervous system; neuropathy; central nervous
     system; CNS; Alzheimer's; Parkinson's disease; Huntington's
     disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
     Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
     arthritis; inflammation; leukaemia; patent; GENESEQ patentdb.
Patent number: WO200153312-A1.
Publication date: 26-JUL-2001.
Filing date: 26-DEC-2000; 2000WO-US034263.
Priority: 23-DEC-1999; 99US-00471275. 21-JAN-2000; 2000US-00488725.
     25-APR-2000; 2000US-00552317. 20-JUN-2000; 2000US-00598042.
     19-JUL-2000; 2000US-00620312. 03-AUG-2000; 2000US-00653450.

14-SEP-2000; 2000US-00662191. 19-OCT-2000; 2000US-00693036.
29-NOV-2000; 2000US-00727344.
Assignee: (HYSE-) HYSEQ INC.
Inventors: Tang YT, Liu C, Asundi V, Chen R, Ma Y, Qian XB, Ren F, Wang D;
    Wang J, Wang Z, Wehrman T, Xu C, Xue AJ, Yang Y, Zhang J, Zhao QA;
    Zhou P, Goodrich R, Drmanac RT;
Cross reference: WPI; 2001-442253/47. N-PSDB; AAI60871.
Title: Novel nucleic acids and polypeptides, useful for treating disorders
    such as central nervous system injuries.
Patent format: Example 2; SEQ ID NO 6646; 10078pp; English.
Comment: The invention relates to human nucleic acids (AAI57798-AAI61369)
    and the encoded polypeptides (AAM38642-AAM42213) with nootropic,
    immunosuppressant and cytostatic activity. The polynucleotides are
    useful in gene therapy. A composition containing a polypeptide or
    polynucleotide of the invention may be used to treat diseases of
    the peripheral nervous system, such as peripheral nervous injuries,
    peripheral neuropathy and localised neuropathies and central
    nervous system diseases, such as Alzheimer's, Parkinson's disease,
    Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager
    Syndrome. Other uses include the utilisation of the activities such
    as: Immune system suppression, Activin/inhibin activity,
    chemotactic/chemokinetic activity, haemostatic and thrombolytic
    activity, cancer diagnosis and therapy, drug screening, assays for
    receptor activity, arthritis and inflammation, leukaemias and C.N.S
    disorders. Note: The sequence data for this patent did not form
    part of the printed specification
Database: GENESEQ patent database (v200420, 23-SEP-2004).

P_AAM39929 Human polypeptide SEQ ID NO 3074 - Homo sapiens.
Length: 234 aa
Accession: P_AAM39929;
Species: Homo sapiens.
Keywords: Human; nootropic; immunosuppressant; cytostatic; gene therapy;
    cancer; peripheral nervous system; neuropathy; central nervous
    system; CNS; Alzheimer's; Parkinson's disease; Huntington's
    disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
    Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
    arthritis; inflammation; leukaemia; patent; GENESEQ patentdb.
Patent number: WO200153312-A1.
Publication date: 26-JUL-2001.
Filing date: 26-DEC-2000; 2000WO-US034263.
Priority: 23-DEC-1999; 99US-00471275. 21-JAN-2000; 2000US-00488725.
    25-APR-2000; 2000US-00552317. 20-JUN-2000; 2000US-00598042.
    19-JUL-2000; 2000US-00620312. 03-AUG-2000; 2000US-00653450.
    14-SEP-2000; 2000US-00662191. 19-OCT-2000; 2000US-00693036.
    29-NOV-2000; 2000US-00727344.
Assignee: (HYSE-) HYSEQ INC.
Inventors: Tang YT, Liu C, Asundi V, Chen R, Ma Y, Qian XB, Ren F, Wang D;
    Wang J, Wang Z, Wehrman T, Xu C, Xue AJ, Yang Y, Zhang J, Zhao QA;
    Zhou P, Goodrich R, Drmanac RT;
Cross reference: WPI; 2001-442253/47. N-PSDB; AAI59085.
Title: Novel nucleic acids and polypeptides, useful for treating disorders
    such as central nervous system injuries.
Patent format: Example 4; SEQ ID NO 3074; 10078pp; English.
Comment: The invention relates to human nucleic acids (AAI57798-AAI61369)
    and the encoded polypeptides (AAM38642-AAM42213) with nootropic,
    immunosuppressant and cytostatic activity. The polynucleotides are

useful in gene therapy. A composition containing a polypeptide or polynucleotide of the invention may be used to treat diseases of the peripheral nervous system, such as peripheral nervous injuries, peripheral neuropathy and localised neuropathies and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager Syndrome. Other uses include the utilisation of the activities such as: Immune system suppression, Activin/inhibin activity, chemotactic/chemokinetic activity, haemostatic and thrombolytic activity, cancer diagnosis and therapy, drug screening, assays for receptor activity, arthritis and inflammation, leukaemias and C.N.S disorders. Note: The sequence data for this patent did not form part of the printed specification
Database: GENESEQ patent database (v200420, 23-SEP-2004).

P_AAU29056 Human PRO polypeptide sequence #33 - Homo sapiens.
Length: 234 aa
Accession: P_AAU29056;
Species: Homo sapiens.
Keywords: PRO polypeptide; mammal; tumour; cancer; human; cattle; horse;
    sheep; dog; cat; pig; goat; rabbit; tumour necrosis factor alpha;
    TNF-alpha; blood; chondrocyte cell; cell proliferation; cell
    differentiation; colon; adrenal; lung; breast; prostate; rectum;
    cervix; liver; genetic disorder; patent; GENESEQ patentdb.
Patent number: WO200168848-A2.
Publication date: 20-SEP-2001.
Filing date: 28-FEB-2001; 2001WO-US006520.
Priority: 01-MAR-2000; 2000WO-US005601. 02-MAR-2000; 2000WO-US005841.
    03-MAR-2000; 2000US-0187202P.01-DEC-2000; 2000WO-US032678.
    20-DEC-2000; 2000WO-US034956. plus 36 more dates.
Assignee: (GETH) GENENTECH INC.
Inventors: Baker KP, Chen J, Desnoyers L, Goddard A, Godowski PJ, Gurney
    AL; Pan J, Smith V, Watanabe CK, Wood WI, Zhang Z;
Cross reference: WPI; 2001-602746/68. N-PSDB; AAS45957.
Title: Novel nucleic acids encoding PRO polypeptides, used to diagnose the
    presence of tumors, such as prostate and breast tumors, in mammals
    and to screen for modulators of the compounds.
Patent format: Claim 11; Fig 66; 774pp; English.
Comment: Sequences AAU29024-AAU29328 represent PRO polypeptides of the
    invention. The PRO polypeptides and their associated nucleic acids
    can be used to detect the presence of a tumour in a mammal by
    comparing the level of expression of a PRO polypeptide in a test
    sample of cells from the animal and a control sample of normal
    cells, whereby a higher level of expression in the test sample
    indicates the presence of a tumour in the mammal. Mammals include
    dogs, cats, cattle, horses, sheep, pigs, goats and rabbits but are
    preferably human. The polypeptides can be used to stimulate tumour
    necrosis factor (TNF) alpha release from human blood, when
    contacted with it. A specific polypeptide can be used to stimulate
    the proliferation or differentiation of chondrocyte cells. The PRO
    proteins can be used to determine the presence of tumours and also
    susceptibility to tumour development, particularly adrenal, lung,
    colon, breast, prostate, rectal, cervical, or liver tumours, in
    mammalian subjects. The oligonucleotide probes specific for the PRO
    nucleic acids can be used for genetic analysis of individuals with
    genetic disorders
Database: GENESEQ patent database (v200420, 23-SEP-2004).

AAH05959 MLN64 N-terminal homolog /pid=AAH05959.1 - Homo sapiens
Length: 234 aa
Species: Homo sapiens (human)
Strausberg,R., Submitted (02-APR-2001) National Institutes of Health,
      Mammalian Gene Collection (MGC), Cancer Genomics Office, National
      Cancer Institute, 31 Center Drive, Room 11A03, Bethesda, MD
      20892-2590, USA Title: Direct Submission
Gene: STARD3NL
Locus: BC005959
Accession: BC005959
Cross-references: LocusID:83930; AAH05959.1; BC005959_1
Database: GBTRANS

AAH03074 MLN64 N-terminal homolog /pid=AAH03074.1 - Homo sapiens
Length: 234 aa
Species: Homo sapiens (human)
Strausberg,R., Submitted (12-FEB-2001) National Institutes of Health,
      Mammalian Gene Collection (MGC), Cancer Genomics Office, National
      Cancer Institute, 31 Center Drive, Room 11A03, Bethesda, MD
      20892-2590, USA Title: Direct Submission
Gene: STARD3NL
Locus: BC003074
Accession: BC003074
Cross-references: LocusID:83930; AAH03074.1; BC003074_1
Database: GBTRANS

NP_114405 MLN64 N-terminal homolog /pid=NP_114405.1 - Homo sapiens
Length: 234 aa
Species: Homo sapiens (human)
Alpy,F., Wendling,C., Rio,M.C. and Tomasetto,C., J. Biol. Chem. 277 (52),
      50780-50787 (2002) Title: MENTHO, a MLN64 homologue devoid of the
      START domain
Gene: STARD3NL
Locus: NM_032016
Accession: NM_032016
Cross-references: LocusID:83930; NP_114405.1; NM_032016_1
Database: REFSEQ

P_AAM39930 Human polypeptide SEQ ID NO 3075 - Homo sapiens.
Length: 216 aa
Accession: P_AAM39930;
Species: Homo sapiens.
Keywords: Human; nootropic; immunosuppressant; cytostatic; gene therapy;
      cancer; peripheral nervous system; neuropathy; central nervous
      system; CNS; Alzheimer's; Parkinson's disease; Huntington's
      disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
      Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
      arthritis; inflammation; leukaemia; patent; GENESEQ patentdb.
Patent number: WO200153312-A1.
Publication date: 26-JUL-2001.
Filing date: 26-DEC-2000; 2000WO-US034263.
Priority: 23-DEC-1999; 99US-00471275. 21-JAN-2000; 2000US-00488725.
      25-APR-2000; 2000US-00552317. 20-JUN-2000; 2000US-00598042.
      19-JUL-2000; 2000US-00620312. 03-AUG-2000; 2000US-00653450.
      14-SEP-2000; 2000US-00662191. 19-OCT-2000; 2000US-00693036.
      29-NOV-2000; 2000US-00727344.

```
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Tue Feb  5 14:46:37 2002 [BLASTN 2.2.1 [Jul-12-2001], NCBI]
/home/ruby/va/Molbio/carpenda/tempids/ss.DNA45409 (1572 bp)
       /home/ruby/va/Molbio/carpenda/tempids/ss.DNA45409
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Sequences producing High-scoring Segment Pairs: Frame Score Match Pct E-val
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   1 P_AAS45957 Human DNA encoding PRO polypeptide seque    +  1572  1572  100   0.0
   2 P_AAF92064 Human PRO1864 cDNA.                         +  1572  1572  100   0.0
   3 AX092282   Sequence 13 from Patent WO0116318.  DNA,    +  1572  1572  100   0.0
   4 P_AAI59085 Human polynucleotide SEQ ID NO 1288.        +  1560  1566  100   0.0
   5 P_AAI60872 Human polynucleotide SEQ ID NO 4861.        +  1560  1566  100   0.0
   6 P_AAI60871 Human polynucleotide SEQ ID NO 4860.        +  1560  1566  100   0.0
   7 BC005959   Homo sapiens, clone MGC:14607 IMAGE:4048    +  1554  1563  100   0.0
   8 BC003074   Homo sapiens, Similar to steroidogenic a    +  1520  1529  100   0.0
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GenBank (Release 143, aug 2004)

1572 100   0.0
P_AAS45957 Human DNA encoding PRO polypeptide sequence #33. 572 bp,
            cDNA, PAT 18-DEC-2001
   ACCESSION   P_AAS45957
   KEYWORDS   GENESEQ; PRO polypeptide; mammal; tumour; cancer; human; cattle;
            horse; sheep; ss; dog; cat; pig; goat; rabbit; tumour necrosis
            factor alpha; TNF-alpha; blood; chondrocyte cell; cell
            proliferation; cell differentiation; colon; adrenal; lung; breast;
            prostate; rectum; cervix; liver; genetic disorder; PCR primer;
            patent; patentdb (v200420, 23-SEP-2004).
   SOURCE   Homo sapiens.
     ORGANISM Homo sapiens.
   REFERENCE   1 (bases 1 to 1572)
     AUTHORS   Baker,K.P., Chen,J., Desnoyers,L., Goddard,A., Godowski,P.J.,
            Gurney,A.L. Pan,J., Smith,V., Watanabe,C.K., Wood,W.I., Zhang,Z.
     TITLE   Novel nucleic acids encoding PRO polypeptides, used to diagnose the
            presence of tumors, such as prostate and breast tumors, in mammals
            and to screen for modulators of the compounds.
     JOURNAL   Patent: WO200168848-A2; Filing Date: 28-FEB-2001; 2001WO-US006520;
            Publication Date: 20-SEP-2001; Priority: 01-MAR-2000;
            2000WO-US005601. 02-MAR-2000; 2000WO-US005841. 03-MAR-2000;
            2000US-0187202P. 06-MAR-2000; 2000US-0186968P. 14-MAR-2000;
            2000US-0189320P. 14-MAR-2000; 2000US-0189328P. 15-MAR-2000;
            2000WO-US006884. 21-MAR-2000; 2000US-0190828P. 21-MAR-2000;
            2000US-0191007P. 21-MAR-2000; 2000US-0191048P. 21-MAR-2000;
            2000US-0191314P. 28-MAR-2000; 2000US-0192655P. 29-MAR-2000;
            2000US-0193032P. 29-MAR-2000; 2000US-0193053P. 30-MAR-2000;
            2000WO-US008439. 04-APR-2000; 2000US-0194449P. 04-APR-2000;
            2000US-0194647P. 11-APR-2000; 2000US-0195975P. 11-APR-2000;
            2000US-0196000P. 11-APR-2000; 2000US-0196187P. 11-APR-2000;
            2000US-0196690P. 11-APR-2000; 2000US-0196820P. 18-APR-2000;
            2000US-0198121P. 18-APR-2000; 2000US-0198585P. 25-APR-2000;
            2000US-0199397P. 25-APR-2000; 2000US-0199550P. 25-APR-2000;
            2000US-0199654P. 03-MAY-2000; 2000US-0201516P. 17-MAY-2000;
            2000WO-US013705. 22-MAY-2000; 2000WO-US014042. 30-MAY-2000;
            2000WO-US014941. 02-JUN-2000; 2000WO-US015264. 05-JUN-2000;
            2000US-0209832P. 28-JUL-2000; 2000WO-US020710. 22-AUG-2000;
            2000US-00644848. 24-AUG-2000; 2000WO-US023328. 08-NOV-2000;
            2000WO-US030952. 01-DEC-2000; 2000WO-US032678. 20-DEC-2000;
            2000WO-US034956; Assignee: (GETH) GENENTECH INC; Cross Reference:

COMMENT   Sequences AAS45925-AAS46231 represent DNA molecules encoding and PCR primers for PRO polypeptides of the invention. The sequences of the invention can be used to detect the presence of a tumour in a mammal by comparing the level of expression of a PRO polypeptide in a test sample of cells from the animal and a control sample of normal cells, whereby a higher level of expression in the test sample indicates the presence of a tumour in the mammal. Mammals include dogs, cats, cattle, horses, sheep, pigs, goats and rabbits but are preferably human. The polypeptides can be used to stimulate tumour necrosis factor (TNF) alpha release from human blood, when contacted with it. A specific polypeptide can be used to stimulate the proliferation or differentiation of chondrocyte cells. The PRO proteins can be used to determine the presence of tumours and also susceptibility to tumour development, particularly adrenal, lung, colon, breast, prostate, rectal, cervical, or liver tumours, in mammalian subjects. The oligonucleotide probes specific for the PRO nucleic acids can be used for genetic analysis of individuals with genetic disorders

FEATURES   Location/Qualifiers
BASE COUNT   446 a   298 c   326 g   502 t
ORIGIN

1572 100   0.0
P_AAF92064   Human PRO1864 cDNA. 572 bp, cDNA, PAT 15-MAY-2001
ACCESSION   P_AAF92064
KEYWORDS   GENESEQ; Human; PRO protein; mapping; patent; patentdb (v200420, 23-SEP-2004).
SOURCE   Homo sapiens.
  ORGANISM   Homo sapiens.
REFERENCE   1   (bases 1 to 1572)
  AUTHORS   Eaton,D.L., Filvaroff,E., Gerritsen,M.E., Goddard,A., Godowski,P.J. Grimaldi,C.J., Gurney,A.L., Watanabe,C.K., Wood,W.I.
  TITLE   Eighty four nucleic acids encoding PRO polypeptides, useful in molecular biology, including use as hybridization probes, and in chromosome and gene mapping.
  JOURNAL   Patent: WO200116318-A2; Filing Date: 24-AUG-2000; 2000WO-US023328; Publication Date: 08-MAR-2001; Priority: 01-SEP-1999; 99WO-US020111. 15-SEP-1999;   99WO-US021090. 07-DEC-1999; 99US-0169495P. 09-DEC-1999;   99US-0170262P. 11-JAN-2000; 2000US-0175481P. 18-FEB-2000; 2000WO-US004341. 18-FEB-2000; 2000WO-US004342. 22-FEB-2000; 2000WO-US004414. 01-MAR-2000; 2000WO-US005601. 03-MAR-2000; 2000US-0187202P. 21-MAR-2000; 2000US-0191007P. 30-MAR-2000; 2000WO-US008439. 25-APR-2000; 2000US-0199397P. 22-MAY-2000; 2000WO-US014042. 05-JUN-2000; 2000US-0209832P; Assignee: (GETH) GENENTECH INC; Cross Reference: WPI; 2001-183260/18. P-PSDB; AAB87532; Patent Format: Claim 2; Fig 13; 278pp; English.
COMMENT   The present sequence is the coding sequence for a human PRO polypeptide (secreted and transmembrane). The PRO protein, and PRO agonists, PRO antagonists or anti-PRO antibodies are useful for preparation of a medicament useful in the treatment of a condition which is responsive to the PRO protein, agonists, antagonists or anti-PRO antibodies. The PRO protein may also be employed as molecular weight markers for protein electrophoresis. The PRO

coding sequence has applications in molecular biology, including
use as hybridisation probes, and in chromosome and gene mapping
FEATURES   Location/Qualifiers
BASE COUNT   446 a   298 c   326 g   502 t
ORIGIN

1572 100   0.0
AX092282   Sequence 13 from Patent WO0116318. 1572 bp,
              DNA, linear, PAT 21-MAR-2001
ACCESSION   AX092282
VERSION   AX092282.1 GI:13444455
KEYWORDS   .
SOURCE   Homo sapiens (human)
   ORGANISM Homo sapiens
             Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
             Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1
   AUTHORS   Eaton,D.L., Filvaroff,E., Gerritsen,M.E., Goddard,A.,
             Godowski,P.J., Grimaldi,C.J., Gurney,A.L., Watanabe,C.K. and
             Wood,W.I.
   TITLE   Secreted and transmembrane polypeptides and nucleic acids encoding
             the same
   JOURNAL   Patent: WO 0116318-A 13 08-MAR-2001;
             Genentech, Inc. (US)
FEATURES   Location/Qualifiers
     source   1..1572
                       /organism="Homo sapiens"
                       /mol_type="unassigned DNA"
                       /db_xref="taxon:9606"
BASE COUNT
ORIGIN

1566 100   0.0
P_AAI59085   Human polynucleotide SEQ ID NO 1288. 675 bp, cDNA, PAT 22-OCT-2001
ACCESSION   P_AAI59085
KEYWORDS   GENESEQ; Human; nootropic; immunosuppressant; cytostatic; gene
             therapy; cancer; peripheral nervous system; neuropathy; central
             nervous system; CNS; Alzheimer's; Parkinson's disease; Huntington's
             disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
             Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
             arthritis; inflammation; leukaemia; patent; patentdb (v200420,
             23-SEP-2004).
SOURCE   Homo sapiens.
   ORGANISM Homo sapiens.
REFERENCE   1 (bases 1 to 1675)
   AUTHORS   Tang,Y.T., Liu,C., Asundi,V., Chen,R., Ma,Y., Qian,X.B.,
             Ren,F., Wang,D. Wang,J., Wang,Z., Wehrman,T., Xu,C., Xue,A.J.,
             Yang,Y., Zhang,J., Zhao,Q.A. Zhou,P., Goodrich,R., Drmanac,R.T.
   TITLE   Novel nucleic acids and polypeptides, useful for treating disorders
             such as central nervous system injuries.
   JOURNAL   Patent: WO200153312-A1; Filing Date: 26-DEC-2000; 2000WO-US034263;
             Publication Date: 26-JUL-2001; Priority: 23-DEC-1999;
             99US-00471275. 21-JAN-2000; 2000US-00488725. 25-APR-2000;
             2000US-00552317. 20-JUN-2000; 2000US-00598042. 19-JUL-2000;
             2000US-00620312. 03-AUG-2000; 2000US-00653450. 14-SEP-2000;
             2000US-00662191. 19-OCT-2000; 2000US-00693036. 29-NOV-2000;
             2000US-00727344; Assignee: (HYSE-) HYSEQ INC; Cross Reference: WPI;

2001-442253/47. P-PSDB; AAM39929; Patent Format: Claim 1; SEQ ID NO
1288; 10078pp; English.

COMMENT   The invention relates to human nucleic acids (AAI57798-AAI61369) and
the encoded polypeptides (AAM38642-AAM42213) with nootropic,
immunosuppressant and cytostatic activity. The polynucleotides are
useful in gene therapy. A composition containing a polypeptide or
polynucleotide of the invention may be used to treat diseases of the
peripheral nervous system, such as peripheral nervous injuries,
peripheral neuropathy and localised neuropathies and central
nervous system diseases, such as Alzheimer's, Parkinson's disease,
Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager
Syndrome. Other uses include the utilisation of the activities such
as: Immune system suppression, Activin/inhibin activity,
chemotactic/chemokinetic activity, haemostatic and thrombolytic
activity, cancer diagnosis and therapy, drug screening, assays for
receptor activity, arthritis and inflammation, leukaemias and C.N.S
disorders. Note: The sequence data for this patent did not form
part of the printed specification

FEATURES   Location/Qualifiers
BASE COUNT   462 a   338 c   361 g   514 t
ORIGIN

1566 100   0.0
P_AAI60872 Human polynucleotide SEQ ID NO 4861. 663 bp, cDNA, PAT 22-OCT-2001
ACCESSION   P_AAI60872
KEYWORDS   GENESEQ; Human; nootropic; immunosuppressant; cytostatic; gene
therapy; cancer; peripheral nervous system; neuropathy; central
nervous system; CNS; Alzheimer's; Parkinson's disease; Huntington's
disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
arthritis; inflammation; leukaemia; patent; patentdb (v200420,
23-SEP-2004).
SOURCE   Homo sapiens.
  ORGANISM Homo sapiens.
REFERENCE   1 (bases 1 to 1663)
  AUTHORS   Tang,Y.T., Liu,C., Asundi,V., Chen,R., Ma,Y., Qian,X.B.,
Ren,F., Wang,D. Wang,J., Wang,Z., Wehrman,T., Xu,C., Xue,A.J.,
Yang,Y., Zhang,J., Zhao,Q.A. Zhou,P., Goodrich,R., Drmanac,R.T.
  TITLE   Novel nucleic acids and polypeptides, useful for treating disorders
such as central nervous system injuries.
  JOURNAL   Patent: WO200153312-A1; Filing Date: 26-DEC-2000; 2000WO-US034263;
Publication Date: 26-JUL-2001; Priority: 23-DEC-1999;
99US-00471275. 21-JAN-2000; 2000US-00488725. 25-APR-2000;
2000US-00552317. 20-JUN-2000; 2000US-00598042. 19-JUL-2000;
2000US-00620312. 03-AUG-2000; 2000US-00653450. 14-SEP-2000;
2000US-00662191. 19-OCT-2000; 2000US-00693036. 29-NOV-2000;
2000US-00727344; Assignee: (HYSE-) HYSEQ INC; Cross Reference: WPI;
2001-442253/47. P-PSDB; AAM41716; Patent Format: Claim 1; SEQ ID NO
4861; 10078pp; English.
COMMENT   The invention relates to human nucleic acids (AAI57798-AAI61369) and
the encoded polypeptides (AAM38642-AAM42213) with nootropic,
immunosuppressant and cytostatic activity. The polynucleotides are
useful in gene therapy. A composition containing a polypeptide or
polynucleotide of the invention may be used to treat diseases of the
peripheral nervous system, such as peripheral nervous injuries,
peripheral neuropathy and localised neuropathies and central
nervous system diseases, such as Alzheimer's, Parkinson's disease,

Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager
Syndrome. Other uses include the utilisation of the activities such
as: Immune system suppression, Activin/inhibin activity,
chemotactic/chemokinetic activity, haemostatic and thrombolytic
activity, cancer diagnosis and therapy, drug screening, assays for
receptor activity, arthritis and inflammation, leukaemias and C.N.S
disorders. Note: The sequence data for this patent did not form
part of the printed specification
FEATURES   Location/Qualifiers
BASE COUNT   454 a   335 c   360 g   514 t
ORIGIN

1566 100   0.0
P_AAI60871 Human polynucleotide SEQ ID NO 4860. 663 bp, cDNA, PAT 22-OCT-2001
ACCESSION   P_AAI60871
KEYWORDS   GENESEQ; Human; nootropic; immunosuppressant; cytostatic; gene
            therapy; cancer; peripheral nervous system; neuropathy; central
            nervous system; CNS; Alzheimer's; Parkinson's disease; Huntington's
            disease; haemostatic; amyotrophic lateral sclerosis; Shy-Drager
            Syndrome; chemotactic; chemokinetic; thrombolytic; drug screening;
            arthritis; inflammation; leukaemia; patent; patentdb (v200420,
            23-SEP-2004).
SOURCE   Homo sapiens.
  ORGANISM Homo sapiens.
REFERENCE   1   (bases 1 to 1663)
  AUTHORS   Tang,Y.T., Liu,C., Asundi,V., Chen,R., Ma,Y., Qian,X.B.,
            Ren,F., Wang,D. Wang,J., Wang,Z., Wehrman,T., Xu,C., Xue,A.J.,
            Yang,Y., Zhang,J., Zhao,Q.A. Zhou,P., Goodrich,R., Drmanac,R.T.
  TITLE   Novel nucleic acids and polypeptides, useful for treating disorders
            such as central nervous system injuries.
  JOURNAL   Patent: WO200153312-A1; Filing Date: 26-DEC-2000; 2000WO-US034263;
            Publication Date: 26-JUL-2001; Priority: 23-DEC-1999;
            99US-00471275. 21-JAN-2000; 2000US-00488725. 25-APR-2000;
            2000US-00552317. 20-JUN-2000; 2000US-00598042. 19-JUL-2000;
            2000US-00620312. 03-AUG-2000; 2000US-00653450. 14-SEP-2000;
            2000US-00662191. 19-OCT-2000; 2000US-00693036. 29-NOV-2000;
            2000US-00727344; Assignee: (HYSE-) HYSEQ INC; Cross Reference: WPI;
            2001-442253/47. P-PSDB; AAM41715; Patent Format: Claim 1; SEQ ID NO
            4860; 10078pp; English.
COMMENT   The invention relates to human nucleic acids (AAI57798-AAI61369) and
            the encoded polypeptides (AAM38642-AAM42213) with nootropic,
            immunosuppressant and cytostatic activity. The polynucleotides are
            useful in gene therapy. A composition containing a polypeptide or
            polynucleotide of the invention may be used to treat diseases of the
            peripheral nervous system, such as peripheral nervous injuries,
            peripheral neuropathy and localised neuropathies and central
            nervous system diseases, such as Alzheimer's, Parkinson's disease,
            Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager
            Syndrome. Other uses include the utilisation of the activities such
            as: Immune system suppression, Activin/inhibin activity,
            chemotactic/chemokinetic activity, haemostatic and thrombolytic
            activity, cancer diagnosis and therapy, drug screening, assays for
            receptor activity, arthritis and inflammation, leukaemias and C.N.S
            disorders. Note: The sequence data for this patent did not form
            part of the printed specification
FEATURES   Location/Qualifiers
BASE COUNT   454 a   335 c   360 g   514 t

ORIGIN

1563 100   0.0
BC005959   Homo sapiens STARD3 N-terminal like, mRNA (cDNA clone MGC:14607
            IMAGE:4048871), complete cds. 1591 bp,
            mRNA, linear, PRI 30-JUN-2004
ACCESSION   BC005959
VERSION   BC005959.1 GI:13543614
KEYWORDS   MGC.
SOURCE   Homo sapiens (human)
  ORGANISM Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1 (bases 1 to 1591)
  AUTHORS   Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G.,
            Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M., Schuler,G.D.,
            Altschul,S.F., Zeeberg,B., Buetow,K.H., Schaefer,C.F., Bhat,N.K.,
            Hopkins,R.F., Jordan,H., Moore,T., Max,S.I., Wang,J., Hsieh,F.,
            Diatchenko,L., Marusina,K., Farmer,A.A., Rubin,G.M., Hong,L.,
            Stapleton,M., Soares,M.B., Bonaldo,M.F., Casavant,T.L.,
            Scheetz,T.E., Brownstein,M.J., Usdin,T.B., Toshiyuki,S.,
            Carninci,P., Prange,C., Raha,S.S., Loquellano,N.A., Peters,G.J.,
            Abramson,R.D., Mullahy,S.J., Bosak,S.A., McEwan,P.J.,
            McKernan,K.J., Malek,J.A., Gunaratne,P.H., Richards,S.,
            Worley,K.C., Hale,S., Garcia,A.M., Gay,L.J., Hulyk,S.W.,
            Villalon,D.K., Muzny,D.M., Sodergren,E.J., Lu,X., Gibbs,R.A.,
            Fahey,J., Helton,E., Ketteman,M., Madan,A., Rodrigues,S.,
            Sanchez,A., Whiting,M., Madan,A., Young,A.C., Shevchenko,Y.,
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            Butterfield,Y.S., Krzywinski,M.I., Skalska,U., Smailus,D.E.,
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  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)
   PUBMED   12477932
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  AUTHORS   Strausberg,R.
  TITLE   Direct Submission
  JOURNAL   Submitted (02-APR-2001) National Institutes of Health, Mammalian
            Gene Collection (MGC), Cancer Genomics Office, National Cancer
            Institute, 31 Center Drive, Room 11A03, Bethesda, MD 20892-2590,
            USA
  REMARK   NIH-MGC Project URL: http://mgc.nci.nih.gov
  COMMENT   Contact: MGC help desk
            Email: cgapbs-r@mail.nih.gov
            Tissue Procurement: ATCC
            cDNA Library Preparation: CLONTECH Laboratories, Inc.
            cDNA Library Arrayed by: The I.M.A.G.E. Consortium (LLNL)
            DNA Sequencing by: Sequencing Group at the Stanford Human Genome
            Center, Stanford University School of Medicine, Stanford, CA 94305
            Web site: http://www-shgc.stanford.edu
            Contact: (Dickson, Mark) mcd@paxil.stanford.edu
            Dickson, M., Schmutz, J., Grimwood, J., Rodriquez, A., and Myers,
            R. M.

            Clone distribution: MGC clone distribution information can be found

through the I.M.A.G.E. Consortium/LLNL at: http://image.llnl.gov
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  AUTHORS    Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G.,
             Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M., Schuler,G.D.,
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            Butterfield,Y.S., Krzywinski,M.I., Skalska,U., Smailus,D.E.,
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  TITLE   Generation and initial analysis of more than 15,000 full-length
            human and mouse cDNA sequences
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)
   PUBMED   12477932
REFERENCE   2 (bases 1 to 1552)
  AUTHORS   Strausberg,R.
  TITLE   Direct Submission
  JOURNAL   Submitted (12-FEB-2001) National Institutes of Health, Mammalian
            Gene Collection (MGC), Cancer Genomics Office, National Cancer
            Institute, 31 Center Drive, Room 11A03, Bethesda, MD 20892-2590,
            USA
  REMARK   NIH-MGC Project URL: http://mgc.nci.nih.gov
COMMENT   On Aug 19, 2003 this sequence version replaced gi:13111773.
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            Email: cgapbs-r@mail.nih.gov
            Tissue Procurement: DCTD/DTP
            cDNA Library Preparation: Rubin Laboratory
            cDNA Library Arrayed by: The I.M.A.G.E. Consortium (LLNL)
            DNA Sequencing by: Institute for Systems Biology
            http://www.systemsbiology.org
            contact: amadan@systemsbiology.org
            Anup Madan, Jessica Fahey, Erin Helton, Mark Ketteman, Anuradha
            Madan, Stephanie Rodrigues, Amy Sanchez and Michelle Whiting

            Clone distribution: MGC clone distribution information can be found
            through the I.M.A.G.E. Consortium/LLNL at: http://image.llnl.gov
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BASE COUNT
ORIGIN

Exhibit 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Eaton, et al.
Appl. No.	:	10/063,557
Filed	:	May 2, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	David J. Blanchard
Group Art Unit	:	1642

DECLARATION OF J. CHRISTOPHER GRIMALDI, UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, J. Christopher Grimaldi, declare and say as follows:

1. I am a Senior Research Associate in the Molecular Biology Department of Genentech, Inc., South San Francisco, CA 94080.

2. I joined Genentech in January of 1999. From 1999 to 2003, I directed the Cloning Laboratory in the Molecular Biology Department. During this time I directed or performed numerous molecular biology techniques including qualitative Polymerase Chain Reaction (PCR) analyses. I am currently involved in, among other projects, the isolation of genes coding for membrane associated proteins which can be used as targets for antibody therapeutics against cancer. In connection with the above-identified patent application, I personally performed or directed the semi-quantitative PCR analyses in the assay entitled "Tumor Versus Normal Differential Tissue Expression Distribution" which is described in EXAMPLE 18 in the specification that were used to identify differences in gene expression between tumor tissue and their normal counterparts.

3. My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).

4. In differential gene expression studies, one looks for genes whose expression levels differ significantly under different conditions, for example, in normal versus diseased tissue.

Chromosomal aberrations, such as gene amplification, and chromosomal translocations are important markers of specific types of cancer and lead to the aberrant expression of specific genes and their encoded polypeptides, including over-expression and under-expression. For example, gene amplification is a process in which specific regions of a chromosome are duplicated, thus creating multiple copies of certain genes that normally exist as a single copy. Gene under-expression can occur when a gene is not transcribed into mRNA. In addition, chromosomal translocations occur when two different chromosomes break and are rejoined to each other chromosome resulting in a chimeric chromosome which displays a different expression pattern relative to the parent chromosomes. Amplification of certain genes such as Her2/Neu [Singleton *et al.*, Pathol. Annu., 27Pt1:165-190], or chromosomal translocations such as t(5;14), [Grimaldi *et al.*, Blood, 73(8):2081-2085(1989); Meeker *et al.*, Blood, 76(2):285-289(1990)] give cancer cells a growth or survival advantage relative to normal cells, and might also provide a mechanism of tumor cell resistance to chemotherapy or radiotherapy. When the chromosomal aberration results in the aberrant expression of a mRNA and the corresponding gene product (the polypeptide), as it does in the aforementioned cases, the gene product is a promising target for cancer therapy, for example, by the therapeutic antibody approach.

5.   Comparison of gene expression levels in normal versus diseased tissue has important implications both diagnostically and therapeutically. For example, those who work in this field are well aware that in the vast majority of cases, when a gene is over-expressed, as evidenced by an increased production of mRNA, the gene product or polypeptide will also be over-expressed. It is unlikely that one identifies increased mRNA expression without associated increased protein expression. This same principle applies to gene under-expression. When a gene is under-expressed, the gene product is also likely to be under-expressed. Stated in another way, two cell samples which have differing mRNA concentrations for a specific gene are expected to have correspondingly different concentration of protein for that gene. Techniques used to detect mRNA, such as Northern Blotting, Differential Display, *in situ* hybridization, quantitative PCR, Taqman, and more recently Microarray technology all rely on the dogma that a change in mRNA will represent a similar change in protein. If this dogma did not hold true then these techniques would have little value and not be so widely used. The use of mRNA quantitation techniques have identified a seemingly endless number of genes which are differentially expressed in various tissues and these genes have subsequently been shown to have correspondingly similar changes in their protein levels. Thus, the detection of increased mRNA expression is expected to result in increased polypeptide expression, and the detection of decreased mRNA expression is expected to result in decreased polypeptide expression. The detection of increased or decreased polypeptide expression can be used for cancer diagnosis and treatment.

6.   However, even in the rare case where the protein expression does not correlate with the mRNA expression, this still provides significant information useful for cancer diagnosis and treatment. For example, if over- or under-expression of a gene product does not correlate with over- or under-expression of mRNA in certain tumor types but does so in others, then identification of both gene expression and protein expression enables more accurate tumor classification and hence better determination of suitable therapy. In addition, absence of over- or

under-expression of the gene product in the presence of a particular over- or under-expression of mRNA is crucial information for the practicing clinician. For example, if a gene is over-expressed but the corresponding gene product is not significantly over-expressed, the clinician accordingly will decide not to treat a patient with agents that target that gene product.

7.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____   Date: ___8 / 10 / 2004___
    J. Christopher Grimaldi

S:\DOCS\AOK\AOK-5479.DOC
071904

Exhibit 3

DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1.   I was awarded a Ph.D. by the Department of Biochemistry of the Michigan State University in 1984. My scientific Curriculum Vitae is attached to and forms part of this Declaration (Exhibit A).

2.   I am currently employed by Genentech, Inc. where my job title is Staff Scientist. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.

3.   As part of the Tumor Antigen Project, my laboratory has been analyzing differential expression of various genes in tumor cells relative to normal cells. The purpose of this research is to identify proteins that are abundantly expressed on certain tumor cells and that are either (i) not expressed, or (ii) expressed at lower levels, on corresponding normal cells. We call such differentially expressed proteins "tumor antigen proteins". When such a tumor antigen protein is identified, one can produce an antibody that recognizes and binds to that protein. Such an antibody finds use in the diagnosis of human cancer and may ultimately serve as an effective therapeutic in the treatment of human cancer.

4.   In the course of the research conducted by Genentech's Tumor Antigen Project, we have employed a variety of scientific techniques for detecting and studying differential gene expression in human tumor cells relative to normal cells, at genomic DNA, mRNA and protein levels. An important example of one such technique is the well known and widely used technique of microarray analysis which has proven to be extremely useful for the identification of mRNA molecules that are differentially expressed in one tissue or cell type relative to another. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor cells at significantly higher levels than in corresponding normal human cells. To date, we have generated antibodies that bind to about 30 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human cancer cells and corresponding normal cells. We have then compared the levels of mRNA and protein in both the tumor and normal cells analyzed.

5.   From the mRNA and protein expression analyses described in paragraph 4 above, we have observed that there is a strong correlation between changes in the level of mRNA present in any particular cell type and the level of protein

expressed from that mRNA in that cell type. In approximately 80% of our observations we have found that increases in the level of a particular mRNA correlates with changes in the level of protein expressed from that mRNA when human tumor cells are compared with their corresponding normal cells.

6.   Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4 and 5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor cell relative to a normal cell typically correlates to a similar increase in abundance of the encoded protein in the tumor cell relative to the normal cell. In fact, it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded protein. While there have been published reports of genes for which such a correlation does not exist, it is my opinion that such reports are exceptions to the commonly understood general rule that increased mRNA levels are predictive of corresponding increased levels of the encoded protein.

7.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: _5/07/04_   By: _Paul Polakis_

Paul Polakis, Ph.D.

SV 2031808 v1

2

CURRICULUM VITAE

PAUL G. POLAKIS
Staff Scientist
Genentech, Inc
1 DNA Way, MS#40
S. San Francisco, CA 94080

EDUCATION:

Ph.D., Biochemistry, Department of Biochemistry,
Michigan State University (1984)

B.S., Biology. College of Natural Science, Michigan State University (1977)

PROFESSIONAL EXPERIENCE:

2002-present	Staff Scientist, Genentech, Inc S. San Francisco, CA
1999- 2002	Senior Scientist, Genentech, Inc., S. San Francisco, CA
1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
1992- 1996	Senior Scientist, Project Leader, Onyx Pharmaceuticals, Richmond, CA
1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, Inc., South SanFrancisco, CA.
1985-1987	Postdoctoral Research Associate, Department of Medicine, Duke University Medical Center, Durham, NC

1984-1985	Assistant Professor, Department of Chemistry, Oberlin College, Oberlin, Ohio
1980-1984	Graduate Research Assistant, Department of Biochemistry, Michigan State University East Lansing, Michigan

PUBLICATIONS:

1. **Polakis, P G.** and Wilson, J. E. 1982 Purification of a Highly Bindable Rat Brain Hexokinase by High Performance Liquid Chromatography. **Biochem. Biophys. Res. Commun.** 107, 937-943.

2. **Polakis, P.G.** and Wilson, J. E. 1984 Proteolytic Dissection of Rat Brain Hexokinase: Determination of the Cleavage Pattern during Limited Digestion with Trypsin. **Arch. Biochem. Biophys.** 234, 341-352.

3. **Polakis, P. G.** and Wilson, J. E. 1985 An Intact Hydrophobic N-Terminal Sequence is Required for the Binding Rat Brain Hexokinase to Mitochondria. **Arch. Biochem. Biophys.** 236, 328-337.

4. Uhing, R.J., **Polakis,P.G.** and Snyderman, R. 1987 Isolaton of GTP-binding Proteins from Myeloid HL60 Cells. **J. Biol. Chem.** 262, 15575-15579.

5. **Polakis, P.G.**, Uhing, R.J. and Snyderman, R. 1988 The Formylpeptide Chemoattractant Receptor Copurifies with a GTP-binding Protein Containing a Distinct 40 kDa Pertussis Toxin Substrate. **J. Biol. Chem.** 263, 4969-4979.

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7. **Polakis, P.G.**, Evans, T. and Snyderman 1989 Multiple Chromatographic Forms of the Formylpeptide Chemoattractant Receptor and their Relationship to GTP-binding Proteins. **Biochem. Biophys. Res. Commun.** 161, 276-283.

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13. Munemitsu, S., Innis, M.A., Clark, R., McCormick, F., Ullrich, A. and **Polakis, P.G.** 1990 Molecular Cloning and Expression of a G25K cDNA, the Human Homolog of the Yeast Cell Cycle Gene CDC42. **Mol. Cell. Biol.** 10, 5977-5982.

14. **Polakis, P.G.** Rubinfeld, B. Evans, T. and McCormick, F. 1991 Purification of Plasma Membrane-Associated GTPase Activating Protein (GAP) Specific for rap-1/krev-1 from HL60 Cells. **Proc. Natl. Acad. Sci. USA** 88, 239-243.

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22. **Polakis P.** and McCormick, F. 1992 Interactions Between p21[ras] Proteins and Their GTPase Activating Proteins. In **Cancer Surveys** (Franks, L. M., ed.) 12, 25-42.

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Exhibit 4

MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

extracts. If these minor cell proteins differ among cells to the same extent as the more abundant proteins, as is commonly assumed, only a small number of protein differences (perhaps several hundred) suffice to create very large differences in cell morphology and behavior.

A Cell Can Change the Expression of Its Genes in Response to External Signals [3]

Most of the specialized cells in a multicellular organism are capable of altering their patterns of gene expression in response to extracellular cues. If a liver cell is exposed to a glucocorticoid hormone, for example, the production of several specific proteins is dramatically increased. Glucocorticoids are released during periods of starvation or intense exercise and signal the liver to increase the production of glucose from amino acids and other small molecules; the set of proteins whose production is induced includes enzymes such as tyrosine aminotransferase, which helps to convert tyrosine to glucose. When the hormone is no longer present, the production of these proteins drops to its normal level.

Other cell types respond to glucocorticoids in different ways. In fat cells, for example, the production of tyrosine aminotransferase is reduced, while some other cell types do not respond to glucocorticoids at all. These examples illustrate a general feature of cell specialization—different cell types often respond in different ways to the same extracellular signal. Underlying this specialization are features that do not change, which give each cell type its permanently distinctive character. These features reflect the persistent expression of different sets of genes.

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein [4]

If differences between the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? There are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the primary RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytoplasm (**RNA transport control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 9–2).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 9–2, only transcriptional control ensures that no superfluous intermediates are synthesized. In the

NUCLEUS CYTOSOL
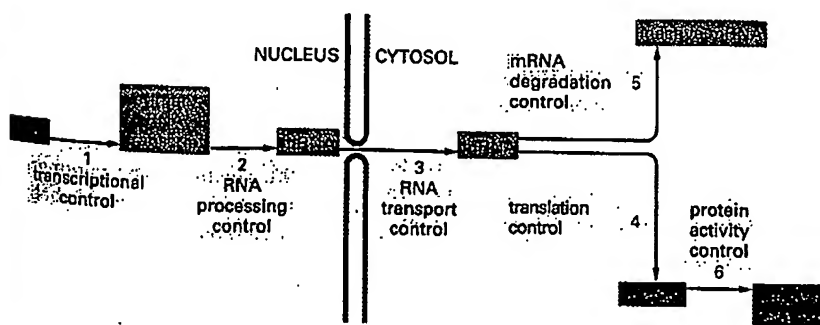
Figure 9–2 Six steps at which eucaryote gene expression can be controlled. Only controls that operate at steps 1 through 5 are discussed in this chapter. The regulation of protein activity (step 6) is discussed in Chapter 5; this includes reversible activation or inactivation by protein phosphorylation as well as irreversible inactivation by proteolytic degradation.

An Overview of Gene Control

following sections we discuss the DNA and protein components that regulate the initiation of gene transcription. We return at the end of the chapter to the other ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-binding Motifs in Gene Regulatory Proteins [5]

How does a cell determine which of its thousands of genes to transcribe? As discussed in Chapter 8, the transcription of each gene is controlled by a regulatory region of DNA near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Other regulatory regions are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices consist of two fundamental types of components: (1) short stretches of DNA of defined sequence and (2) *gene regulatory proteins* that recognize and bind to them.

We begin our discussion of gene regulatory proteins by describing how these proteins were discovered.

Gene Regulatory Proteins Were Discovered Using Bacterial Genetics [6]

Genetic analyses in bacteria carried out in the 1950s provided the first evidence of the existence of **gene regulatory proteins** that turn specific sets of genes on or off. One of these regulators, the *lambda repressor*, is encoded by a bacterial virus, *bacteriophage lambda*. The repressor shuts off the viral genes that code for the protein components of new virus particles and thereby enables the viral genome to remain a silent passenger in the bacterial chromosome, multiplying with the bacterium when conditions are favorable for bacterial growth (see Figure 6–80). The lambda repressor was among the first gene regulatory proteins to be characterized, and it remains one of the best understood, as we discuss later. Other bacterial regulators respond to nutritional conditions by shutting off genes encoding specific sets of metabolic enzymes when they are not needed. The *lac repressor*, for example, the first of these bacterial proteins to be recognized, turns off the production of the proteins responsible for lactose metabolism when this sugar is absent from the medium.

The first step toward understanding gene regulation was the isolation of mutant strains of bacteria and bacteriophage lambda that were unable to shut off specific sets of genes. It was proposed at the time, and later proved, that most of these mutants were deficient in proteins acting as specific repressors for these sets of genes. Because these proteins, like most gene regulatory proteins, are present in small quantities, it was difficult and time-consuming to isolate them. They were eventually purified by fractionating cell extracts on a series of standard chromatography columns (see pp. 166–169). Once isolated, the proteins were shown to bind to specific DNA sequences close to the genes that they
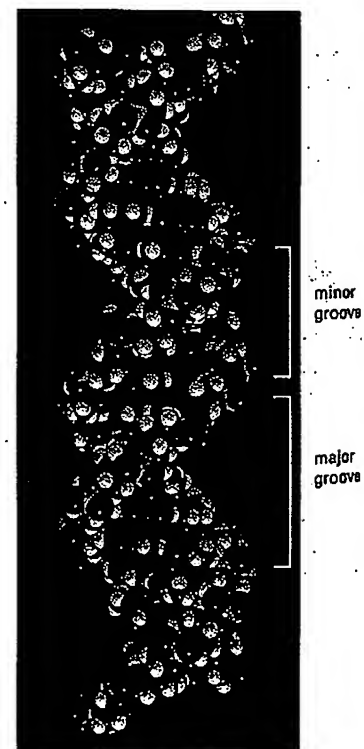
Figure 9–3 Double-helical structure of DNA. The major and minor grooves on the outside of the double helix are indicated. The atoms are colored as follows: carbon, *dark blue;* nitrogen, *light blue;* hydrogen, *white;* oxygen, *red;* phosphorus, *yellow.*
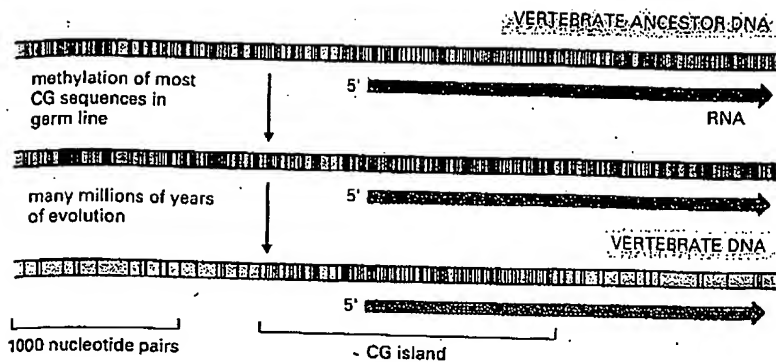
Figure 9–71 A mechanism to explain both the marked deficiency of CG sequences and the presence of CG islands in vertebrate genomes. A *black line* marks the location of an unmethylated CG dinucleotide in the DNA sequence, while a *red line* marks the location of a methylated CG dinucleotide.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides, endowing the cell with a memory of its developmental history. Procaryotes and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms, some of which may be relevant to the creation of specialized cell types in higher eucaryotes. One such mechanism involves a competitive interaction between two (or more) gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory.

In eucaryotes gene transcription is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be expressed in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also utilized by eucaryotic cells to regulate gene expression. In vertebrates DNA methylation also plays a part, mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms.

Posttranscriptional Controls

Although controls on the initiation of gene transcription are the predominant form of regulation for most genes, other controls can act later in the pathway from RNA to protein to modulate the amount of gene product that is made. Although these **posttranscriptional controls**, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than *transcriptional control,* for many genes they are crucial. It seems that every step in gene expression that could be controlled in principle is likely to be regulated under some circumstances for some genes.

We consider the varieties of posttranscriptional regulation in temporal order, according to the sequence of events that might be experienced by an RNA molecule after its transcription has begun (Figure 9–72).
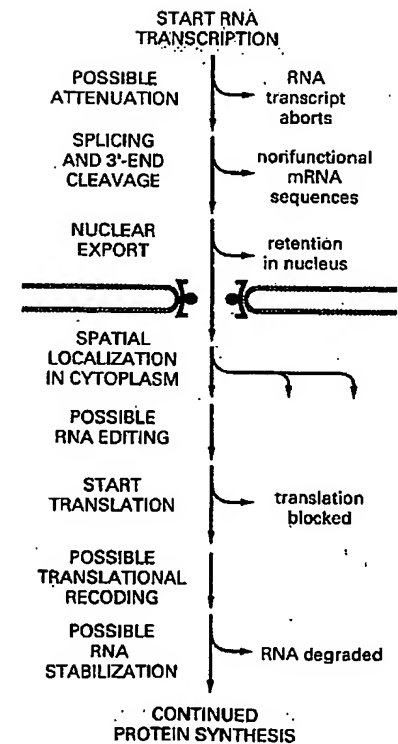
Figure 9–72 Possible posttranscriptional controls on gene expression. Only a few of these controls are likely to be used for any one gene.

Exhibit 5

MOLECULAR BIOLOGY OF
THE CELL

fourth edition

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Front cover Human Genome: Reprinted by permission from *Nature,* International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis,* Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of © Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)
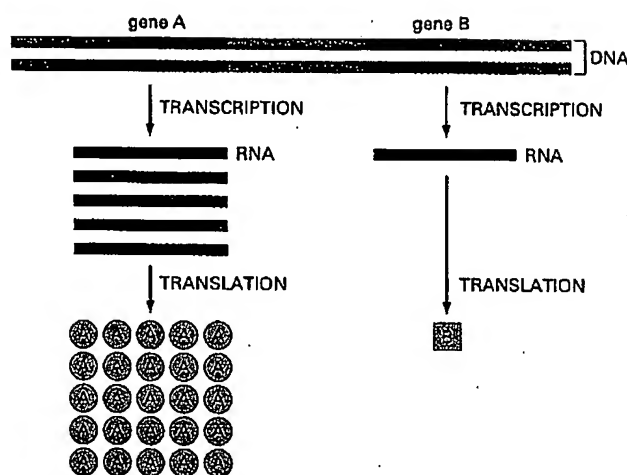
Figure 6–3 Genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. This allows the amount of protein A in the cell to be much greater than that of protein B.

FROM DNA TO RNA

Transcription and translation are the means by which cells read out, or express, the genetic instructions in their genes. Because many identical RNA copies can be made from the same gene, and each RNA molecule can direct the synthesis of many identical protein molecules, cells can synthesize a large amount of protein rapidly when necessary. But each gene can also be transcribed and translated with a different efficiency, allowing the cell to make vast quantities of some proteins and tiny quantities of others (Figure 6–3). Moreover, as we see in the next chapter, a cell can change (or regulate) the expression of each of its genes according to the needs of the moment—most obviously by controlling the production of its RNA.

Portions of DNA Sequence Are Transcribed into RNA

The first step a cell takes in reading out a needed part of its genetic instructions is to copy a particular portion of its DNA nucleotide sequence—a gene—into an RNA nucleotide sequence. The information in RNA, although copied into another chemical form, is still written in essentially the same language as it is in DNA—the language of a nucleotide sequence. Hence the name **transcription**.

Like DNA, RNA is a linear polymer made of four different types of nucleotide subunits linked together by phosphodiester bonds (Figure 6–4). It differs from DNA chemically in two' respects: (1) the nucleotides in RNA are *ribonucleotides*—that is, they contain the sugar ribose (hence the name *ribonucleic acid*) rather than deoxyribose; (2) although, like DNA, RNA contains the bases adenine (A), guanine (G), and cytosine (C), it contains the base uracil (U) instead of the thymine (T) in DNA. Since U, like T, can base-pair by hydrogen-bonding with A (Figure 6–5), the complementary base-pairing properties described for DNA in Chapters 4 and 5 apply also to RNA (in RNA, G pairs with C, and A pairs with U). It is not uncommon, however, to find other types of base pairs in RNA: for example, G pairing with U occasionally.

Despite these small chemical differences, DNA and RNA differ quite dramatically in overall structure. Whereas DNA always occurs in cells as a double-stranded helix, RNA is single-stranded. RNA chains therefore fold up into a variety of shapes, just as a polypeptide chain folds up to form the final shape of a protein (Figure 6–6). As we see later in this chapter, the ability to fold into complex three-dimensional shapes allows some RNA molecules to have structural and catalytic functions.

Transcription Produces RNA Complementary to One Strand of DNA

All of the RNA in a cell is made by DNA transcription, a process that has certain similarities to the process of DNA replication discussed in Chapter 5.

(A) very rare conformational change

(B) infectious seeding of amyloid fiber formation

heterodimer

homodimer

amyloid

(C)

5 nm

(D)
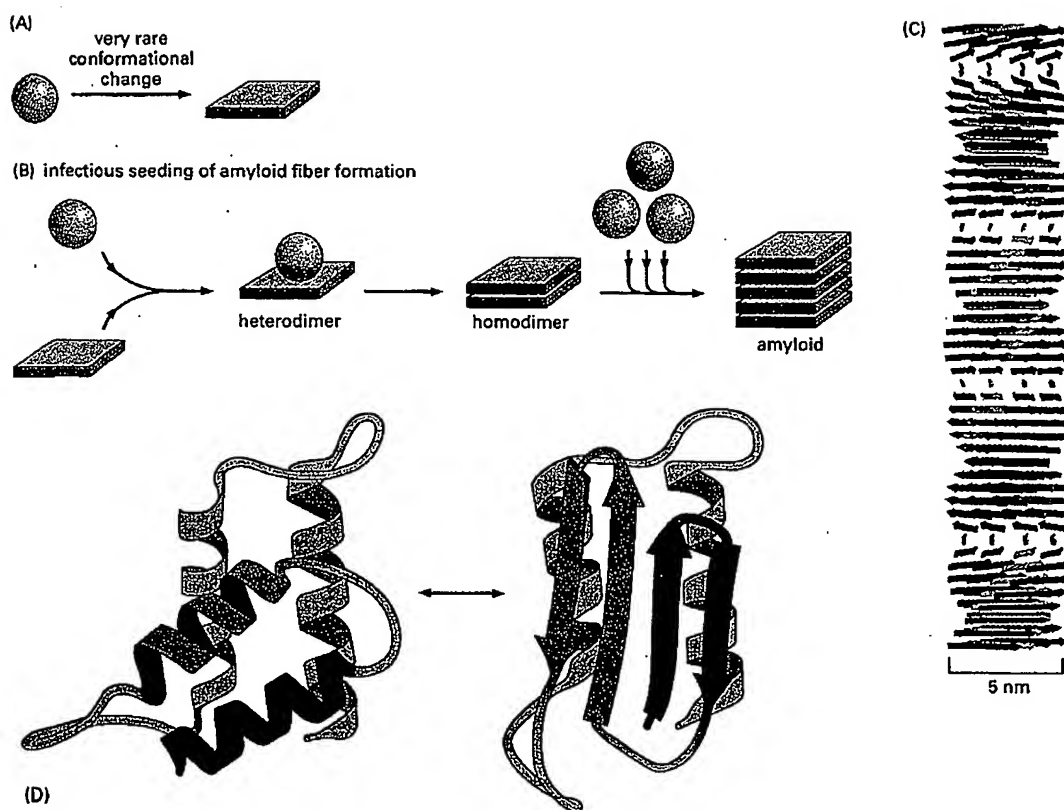
Figure 6–89 Protein aggregates that cause human disease. (A) Schematic illustration of the type of conformational change in a protein that produces material for a cross-beta filament. (B) Diagram illustrating the self-infectious nature of the protein aggregation that is central to prion diseases. PrP is highly unusual because the misfolded version of the protein, called PrP*, induces the normal PrP protein it contacts to change its conformation, as shown. Most of the human diseases caused by protein aggregation are caused by the overproduction of a variant protein that is especially prone to aggregation, but because this structure is not infectious in this way, it cannot spread from one animal to another. (C) Drawing of a cross-beta filament, a common type of protease-resistant protein aggregate found in a variety of human neurological diseases. Because the hydrogen-bond interactions in a β sheet form between polypeptide backbone atoms (see Figure 3–9), a number of different abnormally folded proteins can produce this structure. (D) One of several possible models for the conversion of PrP to PrP*, showing the likely change of two α-helices into four β-strands. Although the structure of the normal protein has been determined accurately, the structure of the infectious form is not yet known with certainty because the aggregation has prevented the use of standard structural techniques. (C, courtesy of Louise Serpell, adapted from M. Sunde et al., J. Mol. Biol. 273:729–739, 1997; D, adapted from S.B. Prusiner, Trends Biochem. Sci. 21:482–487, 1996.)

animals and humans. It can be dangerous to eat the tissues of animals that contain PrP*, as witnessed most recently by the spread of BSE (commonly referred to as the "mad cow disease") from cattle to humans in Great Britain.

Fortunately, in the absence of PrP*, PrP is extraordinarily difficult to convert to its abnormal form. Although very few proteins have the potential to misfold into an infectious conformation, a similar transformation has been discovered to be the cause of an otherwise mysterious "protein-only inheritance" observed in yeast cells.

There Are Many Steps From DNA to Protein

We have seen so far in this chapter that many different types of chemical reactions are required to produce a properly folded protein from the information contained in a gene (Figure 6–90). The final level of a properly folded protein in a cell therefore depends upon the efficiency with which each of the many steps is performed.

We discuss in Chapter 7 that cells have the ability to change the levels of their proteins according to their needs. In principle, any or all of the steps in Fig-
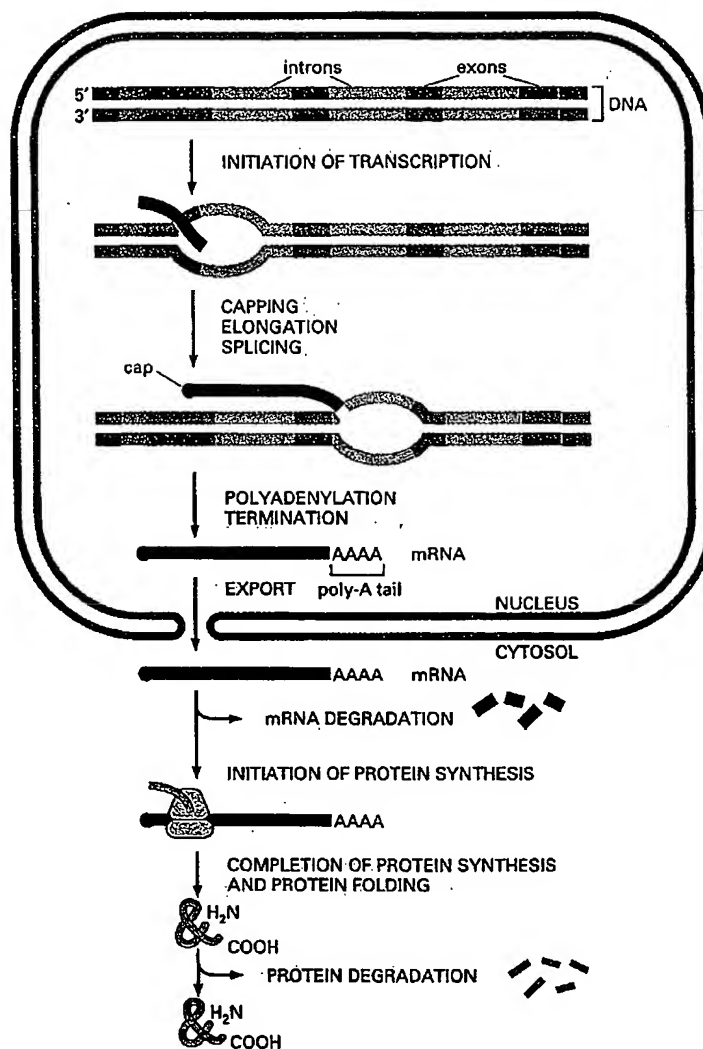
Figure 6–90 The production of a protein by a eucaryotic cell. The final level of each protein in a eucaryotic cell depends upon the efficiency of each step depicted.

ure 6–90) could be regulated by the cell for each individual protein. However, as we shall see in Chapter 7, the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes. This makes sense, inasmuch as the most efficient way to keep a gene from being expressed is to block the very first·step—the transcription of its DNA sequence into an RNA molecule.

Summary

The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome. The amino acids used for protein synthesis are first attached to a family of tRNA molecules, each of which recognizes, by complementary base-pair interactions, particular sets of three nucleotides in the mRNA (codons). The sequence of nucleotides in the mRNA is then read from one end to the other in sets of three according to the genetic code.

To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. A large ribosomal subunit binds to complete the ribosome and begin the elongation phase of protein synthesis. During this phase, aminoacyl tRNAs—each bearing a specific amino acid bind sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential
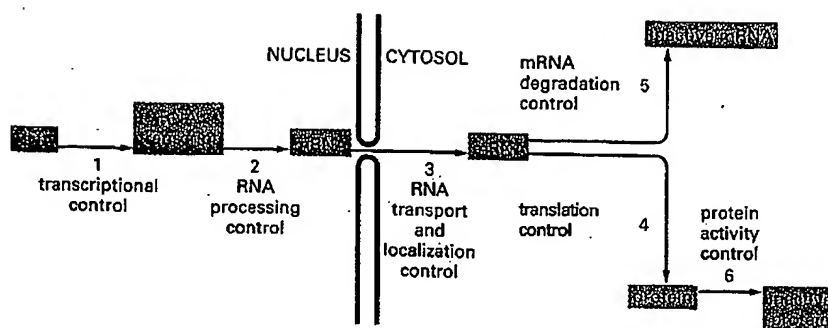
Figure 7–5 Six steps at which eucaryotic gene expression can be controlled. Controls that operate at steps 1 through 5 are discussed in this chapter. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation (discussed in Chapter 3) as well as irreversible inactivation by proteolytic degradation (discussed in Chapter 6).

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein

If differences among the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? As we saw in the last chapter, there are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where in the cytosol they are localized (**RNA transport and localization control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 7–5).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 7–5, only transcriptional control ensures that the cell will not synthesize superfluous intermediates. In the following sections we discuss the DNA and protein components that perform this function by regulating the initiation of gene transcription. We shall return at the end of the chapter to the additional ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-BINDING MOTIFS IN GENE REGULATORY PROTEINS

How does a cell determine which of its thousands of genes to transcribe? As mentioned briefly in Chapters 4 and 6, the transcription of each gene is controlled by a regulatory region of DNA relatively near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Many others are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices

occur in the germ line, the cell lineage that gives rise to sperm or eggs. Most of the DNA in vertebrate germ cells is inactive and highly methylated. Over long periods of evolutionary time, the methylated CG sequences in these inactive regions have presumably been lost through spontaneous deamination events that were not properly repaired. However promoters of genes that remain active in the germ cell lineages (including most housekeeping genes) are kept unmethylated, and therefore spontaneous deaminations of Cs that occur within them can be accurately repaired. Such regions are preserved in modern day vertebrate cells as CG islands. In addition, any mutation of a CG sequence in the genome that destroyed the function or regulation of a gene in the adult would be selected against, and some CG islands are simply the result of a higher than normal density of critical CG sequences.

The mammalian genome contains an estimated 20,000 CG islands. Most of the islands mark the 5′ ends of transcription units and thus, presumably, of genes. The presence of CG islands often provides a convenient way of identifying genes in the DNA sequences of vertebrate genomes.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character through many cell division cycles and even when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides. These features endow the cell with a memory of its developmental history. Bacteria and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms. One such mechanism involves a competitive interaction between two gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory. Negative feedback loops with programmed delays form the basis for cellular clocks.

In eucaryotes the transcription of a gene is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be active in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also used by eucaryotic cells to regulate gene expression. An especially dramatic case is the inactivation of an entire X chromosome in female mammals. In vertebrates DNA methylation also functions in gene regulation, being used mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms. DNA methylation also underlies the phenomenon of genomic imprinting in mammals, in which the expression of a gene depends on whether it was inherited from the mother or the father.

POSTTRANSCRIPTIONAL CONTROLS

In principle, every step required for the process of gene expression could be controlled. Indeed, one can find examples of each type of regulation, although any one gene is likely to use only a few of them. Controls on the initiation of gene transcription are the predominant form of regulation for most genes. But other controls can act later in the pathway from DNA to protein to modulate the amount of gene product that is made. Although these posttranscriptional controls, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than transcriptional control, for many genes they are crucial.
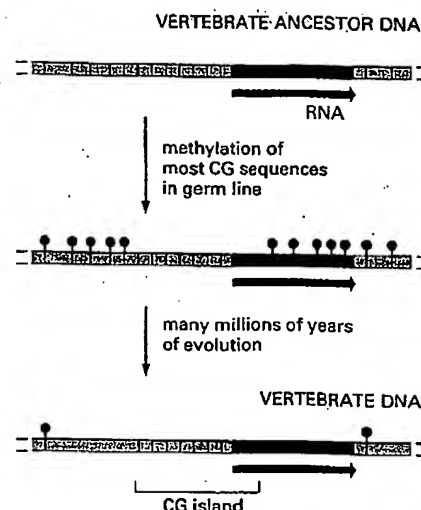

VERTEBRATE ANCESTOR DNA

RNA

methylation of most CG sequences in germ line

many millions of years of evolution

VERTEBRATE DNA

CG island

Figure 7–86 A mechanism to explain both the marked overall deficiency of CG sequences and their clustering into CG islands in vertebrate genomes. A *black line* marks the location of a CG dinucleotide in the DNA sequence, while a *red* "lollipop" indicates the presence of a methyl group on the CG dinucleotide. CG sequences that lie in regulatory sequences of genes that are transcribed in germ cells are unmethylated and therefore tend to be retained in evolution. Methylated CG sequences, on the other hand, tend to be lost through deamination of 5-methyl C to T, unless the CG sequence is critical for survival.

Exhibit 6

CHAPTER 29

Regulation of transcription

Genes VI (1997) CH 29, pp. 847-848.
Benjamin Lewin

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins, that is, those transcribed by RNA polymerase II. In principle, the expression of these genes might be regulated at any one of several stages. The concept of the "level of control" implies that gene expression is not necessarily an automatic process once it has begun. It could be regulated in a gene-specific way at any one of several sequential steps. We can distinguish (at least) five potential control points, forming the series:

Activation of gene structure
↓
Initiation of transcription
↓
Processing the transcript
↓
Transport to cytoplasm
↓
Translation of mRNA

The existence of the first step is implied by the discovery that genes may exist in either of two structural conditions. Relative to the state of most of the genome, genes are found in an "active" state in the cells in which they are expressed (see Chapter 27). The change of structure is distinct from the act of transcription, and indicates that the gene is "transcribable." This suggests that acquisition of the "active" structure must be the first step in gene expression.

Transcription of a gene in the active state is controlled at the stage of initiation, that is, by the interaction of RNA polymerase with its promoter. This is now becoming susceptible to analysis in the *in vitro* systems (see Chapter 28). For most genes, this is a major control point; probably it is the most common level of regulation.

There is at present no evidence for control at subsequent stages of transcription in eukaryotic cells, for example, via antitermination mechanisms.

The primary transcript is modified by capping at the 5' end, and usually also by polyadenylation at the 3' end. Introns must be spliced out from the transcripts of interrupted genes. The mature RNA must be exported from the nucleus to the cytoplasm. Regulation of gene expression by selection of sequences at the level of nuclear RNA might involve any or all of these stages, but the one for which we have most evidence concerns changes in splicing: some genes are expressed by means of alternative splicing patterns whose regulation controls the type of protein product (see Chapter 30).

Finally, the translation of an mRNA in the cytoplasm can be specifically controlled. There is little evidence for the employment of this mechanism in adult somatic cells, but it does occur in some embryonic situations, as described in Chapter 7. The mechanism is presumed to involve the blocking of initiation of translation of some mRNAs by specific protein factors.

But having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein, it is clear

that the overwhelming majority of regulatory events occur at the initiation of transcription. Regulation of tissue-specific gene transcription lies at the heart of eukaryotic differentiation; indeed, we see examples in Chapter 38 in which proteins that regulate embryonic development prove to be transcription factors. A regulatory transcription factor serves to provide common control of a large number of target genes, and we seek to answer two questions about this mode of regulation: what identifies the common target genes to the transcription factor; and how is the activity of the transcription factor itself regulated in response to intrinsic or extrinsic signals?

Response elements identify genes under common regulation

The principle that emerges from characterizing groups of genes under common control is that *they share a promoter element that is recognized by a regulatory transcription factor.* An element that causes a gene to respond to such a factor is called a response element; examples are the HSE (heat shock response element), GRE (glucocorticoid response element), SRE (serum response element).

The properties of some inducible transcription factors and the elements that they recognize are summarized in Table 29.1. Response elements have the same general characteristics as upstream elements of promoters or enhancers. They contain short consensus sequences, and copies of the response elements found in different genes are closely related, but not necessarily identical. The region bound by the factor extends for a short distance on either side of the consensus sequence. In promoters, the elements are not present at fixed distances from the startpoint, but are usually <200 bp upstream of it. The presence of a single element usually is sufficient to confer the regulatory response, but sometimes there are multiple copies.

Response elements may be located in promoters or in enhancers. Some types of elements are typically found in one rather than the other: usually an HSE is found in a promoter, while a GRE is found in an enhancer. We assume that all response elements function by the same general principle. *A gene is regulated by a sequence at the promoter or enhancer that is recognized by a specific protein. The protein functions as a transcription factor needed for RNA polymerase to initiate.* Active protein is available only under conditions when the gene is to be expressed; its absence means that the promoter is not activated by this particular circuit.

An example of a situation in which many genes are controlled by a single factor is provided by the heat shock response. This is common to a wide range of prokaryotes and eukaryotes and involves multiple controls of gene expression: an increase in temperature turns off transcription of some genes, turns on transcription of the heat shock genes, and causes changes in the translation of mRNAs. The control of the heat shock genes illustrates the differences between prokaryotic and eukaryotic modes of control. In bacteria, a new sigma factor is synthesized that directs RNA polymerase holoenzyme to recognize an alter-

Regulatory Agent	Module	Consensus	Factor
Heat shock	HSE	CNNGAANNTCCNNG	HSTF
Glucocorticoid	GRE	TGGTACAAATGTTCT	Receptor
Phorbol ester	TRE	TGACTCA	AP1
Serum	SRE	CCATATTAGG	SRF

Exhibit 7

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Research

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Prostate stem cell antigen (PSCA) expression in human prostate cancer tissues and its potential role in prostate carcinogenesis and progression of prostate cancer

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Abstract

Background: Prostate stem cell antigen (PSCA) is a recently defined homologue of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. The purpose of the present study was to examine the expression status of PSCA protein and mRNA in clinical specimens of human prostate cancer (Pca) and to validate it as a potential molecular target for diagnosis and treatment of Pca.

Materials and Methods: Immunohistochemical (IHC) and *in situ* hybridization (ISH) analyses of PSCA expression were simultaneously performed on paraffin-embedded sections from 20 benign prostatic hyperplasia (BPH), 20 prostatic intraepithelial neoplasm (PIN) and 48 prostate cancer (Pca) tissues, including 9 androgen-independent prostate cancers. The level of PSCA expression was semiquantitatively scored by assessing both the percentage and intensity of PSCA-positive staining cells in the specimens. Then compared PSCA expression between BPH, PIN and Pca tissues and analysed the correlations of PSCA expression level with pathological grade, clinical stage and progression to androgen-independence in Pca.

Results: In BPH and low grade PIN, PSCA protein and mRNA staining were weak or negative and less intense and uniform than that seen in HGPIN and Pca. There were moderate to strong PSCA protein and mRNA expression in 8 of 11 (72.7%) HGPIN and in 40 of 48 (83.4%) Pca specimens examined by IHC and ISH analyses, with statistical significance compared with BPH (20%) and low grade PIN (22.2%) samples (p < 0.05, respectively). The expression level of PSCA increased with high Gleason grade, advanced stage and progression to androgen-independence (p < 0.05, respectively). In addition, IHC and ISH staining showed a high degree of correlation between PSCA protein and mRNA overexpression.

Conclusions: Our data demonstrate that PSCA as a new cell surface marker is overexpressed by a majority of human Pca. PSCA expression correlates positively with adverse tumor characteristics, such as increasing pathological grade (poor cell differentiation), worsening clinical stage and androgen-independence, and speculatively with prostate carcinogenesis. PSCA protein overexpression results from upregulated transcription of PSCA mRNA. PSCA may have prognostic utility and may be a promising molecular target for diagnosis and treatment of Pca.

Introduction

Prostate cancer (Pca) is the second leading cause of cancer-related death in American men and is becoming a common cancer increasing in China. Despite recently great progress in the diagnosis and management of localized disease, there continues to be a need for new diagnostic markers that can accurately discriminate between indolent and aggressive variants of Pca. There also continues to be a need for the identification and characterization of potential new therapeutic targets on Pca cells. Current diagnostic and therapeutic modalities for recurrent and metastatic Pca have been limited by a lack of specific target antigens of Pca.

Although a number of prostate-specific genes have been identified (i.e. prostate specific antigen, prostatic acid phosphatase, glandular kallikrein 2), the majority of these are secreted proteins not ideally suited for many immunological strategies. So, the identification of new cell surface antigens is critical to the development of new diagnostic and therapeutic approaches to the management of Pca.

Reiter RE et al [1] reported the identification of prostate stem cell antigen (PSCA), a cell surface antigen that is predominantly prostate specific. The PSCA gene encodes a 123 amino acid glycoprotein, with 30% homology to stem cell antigen 2 (Sca 2). Like Sca-2, PSCA also belongs to a member of the Thy-1/Ly-6 family and is anchored by a glycosylphosphatidylinositol (GPI) linkage. mRNA *in situ* hybridization (ISH) localized PSCA expression in normal prostate to the basal cell epithelium, the putative stem cell compartment of prostatic epithelium, suggesting that PSCA may be a marker of prostate stem/progenitor cells.

In order to examine the status of PSCA protein and mRNA expression in human Pca and validate it as a potential diagnostic and therapeutic target for Pca, we used immunohistochemistry (IHC) and *in situ* hybridization (ISH) simultaneously, and conducted PSCA protein and mRNA expression analyses in paraffin-embedded tissue specimens of benign prostatic hyperplasia (BPH, n = 20), prostate intraepithelial neoplasm (PIN, n = 20) and prostate cancer (Pca, n = 48). Furthermore, we evaluated the possible correlation of PSCA expression level with Pca tumorigenesis, grade, stage and progression to androgen-independence.

Materials and methods

Tissue samples

All of the clinical tissue specimens studied herein were obtained from 80 patients of 57–84 years old by prostatectomy, transurethral resection of prostate (TURP) or biopsies. The patients were classified as 20 cases of BPH, 20 cases of PIN, 40 cases of primary Pca, including 9 patients

with recurrent Pca and a history of androgen ablation therapy (orchiectomy and/or hormonal therapy), who were referred to as androgen-independent prostate cancers. Eight specimens were harvested from these androgen-independent Pca patients prior to androgen ablation treatment. Each tissue sample was cut into two parts, one was fixed in 10% formalin for IHC and the other treated with 4% paraformaldehyde/0.1 M PBS PH 7.4 in 0.1% DEPC for 1 h for ISH analysis, and then embedded in paraffin. All paraffin blocks examined were then cut into 5 μm sections and mounted on the glass slides specific for IHC and ISH respectively in the usual fashion. H&E-stained section of each Pca was evaluated and assigned a Gleason score by the experienced urological pathologist at our institution based on the criteria of Gleason score [2]. The Gleason sums are summarized in Table 1. Clinical staging was performed according to Jewett-whitmore-prout staging system, as shown in Table 2. In the category of PIN, we graded the specimens into two groups, i.e. low grade PIN (grade I – II) and high grade PIN (HGPIN, grade III) on the basis of literatures [3,4].

Immunohistochemical (IHC) analysis

Briefly, tissue sections were deparaffinized, dehydrated, and subjected to microwaving in 10 mmol/L citrate buffer, PH 6.0 (Boshide, Wuhan, China) in a 900 W oven for 5 min to induce epitope retrieval. Slides were allowed to cool at room temperature for 30 min. A primary mouse antibody specific to human PSCA (Boshide, Wuhan, China) with a 1:100 dilution was applied to incubate with the slides at room temperature for 2 h. Labeling was detected by sequentially adding biotinylated secondary antibodies and strepavidin-peroxidase, and localized using 3,3'-diaminobenzidine reaction. Sections were then counterstained with hematoxylin. Substitution of the primary antibody with phosphate-buffered-saline (PBS) served as a negative-staining control.

mRNA in situ hybridization (ISH)

Five-μm-thick tissue sections were deparaffinized and dehydrated, then digested in pepsin solution (4 mg/ml in 3% citric acid) for 20 min at 37.5°C, and further processed for ISH. Digoxigenin-labeled sense and antisense human PSCA RNA probes (obtained from Boshide, Wuhan, China) were hybridized to the sections at 48°C overnight. The posthybridization wash with a high stringency was performed sequentially at 37°C in 2 × standard saline citrate (SSC) for 10 min, in 0.5 × SSC for 15 min and in 0.2 × SSC for 30 min. The slides were then incubated to biotinylated mouse anti-digoxigenin antibody at 37.5°C for 1 h followed by washing in 1 × PBS for 20 min at room temperature, and then to strepavidin-peroxidase at 37.5°C for 20 min followed by washing in 1 × PBS for 15 min at room temperature. Subsequently, the slides were developed with diaminobenzidine and then coun-

Table 1: Correlation of PSCA expression with Gleason score

Gleason score	Intensity × frequency	
	0–6 (%)	9 (%)
2–4	5 (83)	1 (17)
5–7	19 (79)	5 (21)
8–10	5 (28)	13 (72)

Table 2: Correlation of PSCA expression with clinical stage

Tumor stage	Intensity × frequency	
	0–6 (%)	9 (%)
≤B	27 (67.5)	13 (32.5)
≥C	2 (25)	6 (75)

terstained with hematoxylin to localize the hybridization signals. Sections hybridized with the sense control probes routinely did not show any specific hybridization signal above background. All slides were hybridized with PBS to substitute for the probes as a negative control.

Scoring methods

To determine the correlation between the results of PSCA immunostaining and mRNA *in situ* hybridization, the same scoring manners are taken in the present study for PSCA protein staining by IHC and PSCA mRNA staining by ISH. Each slide was read and scored by two independently experienced urological pathologists using Olympus BX-41 light microscopes. The evaluation was done in a blinded fashion. For each section, five areas of similar grade were analyzed semiquantitatively for the fraction of cells staining. Fifty percent of specimens were randomly chosen and rescored to determine the degree of interobserver and intraobserver concordance. There was greater than 95% intra- and interobserver agreement.

The intensity of PSCA expression evaluated microscopically was graded on a scale of 0 to 3+ with 3 being the highest expression observed (0, no staining; 1+, mildly intense; 2+, moderately intense; 3+, severely intense). The staining density was quantified as the percentage of cells staining positive for PSCA with the primary antibody or hybridization probe, as follows: 0 = no staining; 1 = positive staining in <25% of the sample; 2 = positive staining in 25%–50% of the sample; 3 = positive staining in >50%

of the sample. Intensity score (0 to 3+) was multiplied by the density score (0–3) to give an overall score of 0–9 [1,5]. In this way, we were able to differentiate specimens that may have had focal areas of increased staining from those that had diffuse areas of increased staining [6]. The overall score for each specimen was then categorically assigned to one of the following groups: 0 score, negative expression; 1–2 scores, weak expression; 3–6 scores, moderate expression; 9 score, strong expression.

Statistical analysis

Intensity and density of PSCA protein and mRNA expression in BPH, PIN and Pca tissues were compared using the Chi-square and Student's *t*-test. Univariate associations between PSCA expression and Gleason score, clinical stage and progression to androgen-independence were calculated using Fisher's Exact Test. For all analyses, p < 0.05 was considered statistically significant.

Results

PSCA expression in BPH

In general, PSCA protein and mRNA were expressed weakly in individual samples of BPH. Some areas of prostate expressed weak levels (composite score 1–2), whereas other areas were completely negative (composite score 0). Four cases (20%) of BPH had moderate expression of PSCA protein and mRNA (composite score 4–6) by IHC and ISH. In 2/20 (10%) BPH specimens, PSCA mRNA expression was moderate (composite score 3–6), but PSCA protein expression was weak (composite score

2) in one and negative (composite score 0) in the other. PSCA expression was localized to the basal and secretory epithelial cells, and prostatic stroma was almost negative staining for PSCA protein and mRNA in all cases examined.

PSCA expression in PIN

In this study, we detected weak or negative expression of PSCA protein and mRNA (≤ 2 scores) in 7 of 9 (77.8%) low grade PIN and in 2 of 11 (18.2%) HGPIN, and moderate expression (3–6 scores) in the rest 2 low grade PIN and 5 of 11 (45.5%) HGPIN. One HGPIN with moderate PSCA mRNA expression (6 score) was found weak staining for PSCA protein (2 score) by IHC. Strong PSCA protein and mRNA expression (9 score) were detected in the remaining 3 of 11 (27.3%) HGPIN. There was a statistically significant difference of PSCA protein and mRNA expression levels observed between HGPIN and BPH ($p < 0.05$), but no statistical difference reached between low grade PIN and BPH ($p > 0.05$).

PSCA expression in Pca

In order to determine if PSCA protein and mRNA can be detected in prostate cancers and if PSCA expression levels are increased in malignant compared with benign glands, Forty-eight paraffin-embedded Pca specimens were analysed by IHC and ISH. It was shown that 19 of 48 (39.6%) Pca samples stained very strongly for PSCA protein and mRNA with a score of 9 and another 21 (43.8%) specimens displayed moderate staining with scores of 4–6 (Figure 1). In addition, 4 specimens with moderate to strong PSCA mRNA expression (scores of 4–9) had weak protein staining (a score of 2) by IHC analyses. Overall, Pca expressed a significantly higher level of PSCA protein and mRNA than any other specimen category in this study ($p < 0.05$, compared with BPH and PIN respectively). The result demonstrates that PSCA protein and mRNA are overexpressed by a majority of human Pca.

Correlation of PSCA expression with Gleason score in Pca

Using the semi-quantitative scoring method as described in Materials and Methods, we compared the expression level of PSCA protein and mRNA with Gleason grade of Pca, as shown in Table 1. Prostate adenocarcinomas were graded by Gleason score as 2–4 scores = well-differentiation, 5–7 scores = moderate-differentiation and 8–10 scores = poor-differentiation [7]. Seventy-two percent of Gleason scores 8–10 prostate cancers had very strong staining of PSCA compared to 21% with Gleason scores 5–7 and 17% with 2–4 respectively, demonstrating that poorly differentiated Pca had significantly stronger expression of PSCA protein and mRNA than moderately and well differentiated tumors ($p < 0.05$). As depicted in Figure 1, IHC and ISH analyses showed that PSCA protein and mRNA expression in several cases of poorly differen-

tiated Pca were particularly prominent, with more intense and uniform staining. The results indicate that PSCA expression increases significantly with higher tumor grade in human Pca.

Correlation of PSCA expression with clinical stage in Pca

With regards to PSCA expression in every stage of Pca, we showed the results in Table 2. Seventy-five percent of locally advanced and node positive cancers (i.e. C-D stages) expressed statistically high levels of PSCA versus 32.5% that were organ confined (i.e. A-B stages) ($p < 0.05$). The data demonstrate that PSCA expression increases significantly with advanced tumor stage in human Pca.

Correlation of PSCA expression with androgen-independent progression of Pca

All 9 specimens of androgen-independent prostate cancers stained positive for PSCA protein and mRNA. Eight specimens were obtained from patients managed prior to androgen ablation therapy. Seven of eight (87.5%) of these androgen-independent prostate cancers were in the strongest staining category (score = 9), compared with three out of eight (37.5%) of patients with androgen-dependent cancers ($p < 0.05$). The results demonstrate that PSCA expression increases significantly with progression to androgen-independence of human Pca.

It is evident from the results above that within a majority of human prostate cancers the level of PSCA protein and mRNA expression correlates significantly with increasing grade, worsening stage and progression to androgen-independence.

Correlation of PSCA immunostaining and mRNA in situ hybridization

In all 88 specimens surveyed herein, we compared the results of PSCA IHC staining with mRNA ISH analysis. Positive staining areas and its intensity and density scores evaluated by IHC were identical to those seen by ISH in 79 of 88 (89.8%) specimens (18/20 BPH, 19/20 PIN and 42/48 Pca respectively). Importantly, 27/27 samples with PSCA mRNA composite scores of 0–2, 32/36 samples with scores of 3–6 and 22/24 samples with a score of 9 also had PSCA protein expression scores of 0–2, 3–6 and 9 respectively. However, in 5 samples with PSCA mRNA overall scores of 3–6 and in 2 with scores of 9 there were less or negative PSCA protein expression (i.e. scores of 0–4), suggesting that this may reflect posttranscriptional modification of PSCA or that the epitopes recognized by PSCA mAb may be obscured in some cancers. The data demonstrate that the results of PSCA immunostaining were consistent with those of mRNA ISH analysis, showing a high degree of correlation between PSCA protein and mRNA expression.
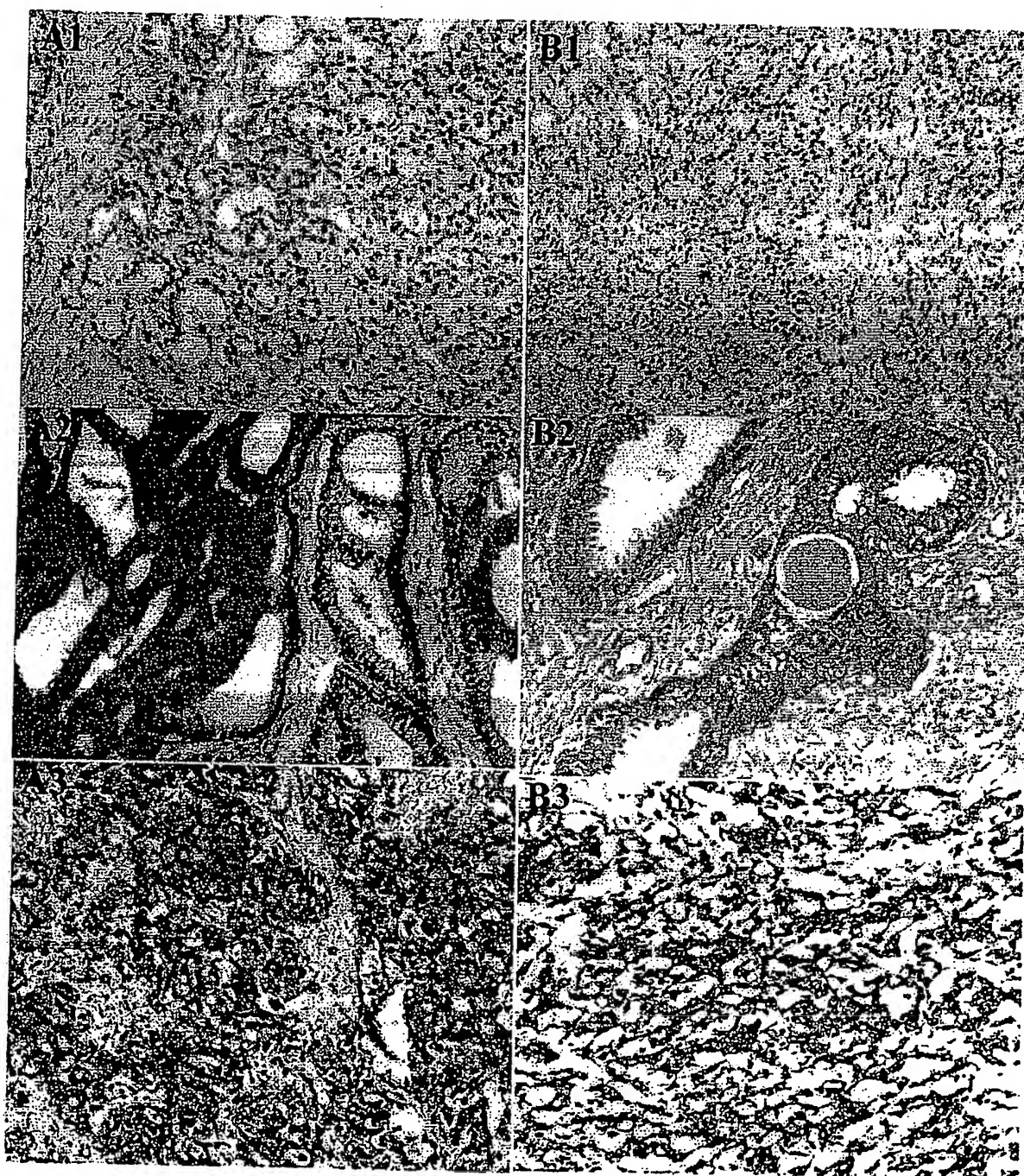
Figure 1
Representatives of PSCA IHC and ISH staining in Pca (A. IHC staining, B. ISH staining, ×200 magnification). A_1, B_1: negative control of IHC and ISH. PBS replacing the primary antibody (A_1) and hybridization with a sense PSCA probe (B_1) showed no background staining. A_2, B_2: a moderately differentiated Pca (Gleason score = 3+3 = 6) with moderate staining (composite score = 6) in all malignant cells; A_2: IHC shows not only cell surface but also apparent cytoplasmic staining of PSCA protein. A_3, B_3: a poorly differentiated Pca (Gleason score = 4+4 = 8) with very strong staining (composite score = 9) in all malignant cells.

Discussion

PSCA is homologous to a group of cell surface proteins that mark the earliest phase of hematopoietic development. PSCA mRNA expression is prostate-specific in normal male tissues and is highly up-regulated in both androgen-dependent and-independent Pca xenografts (LAPC-4 tumors). We hypothesize that PSCA may play a role in Pca tumorigenesis and progression, and may serve as a target for Pca diagnosis and treatment. In this study, IHC and ISH showed that in general there were weak or absent PSCA protein and mRNA expression in BPH and low grade PIN tissues. However, PSCA protein and mRNA are widely expressed in HGPIN, the putative precursor of invasive Pca, suggesting that up-regulation of PSCA is an early event in prostate carcinogenesis. Recently, Reiter RE et al [1], using ISH analysis, reported that 97 of 118 (82%) HGPIN specimens stained strongly positive for PSCA mRNA. A very similar finding was seen on mouse PSCA (mPSCA) expression in mouse HGPIN tissues by Tran C. P et al [8]. These data suggest that PSCA may be a new marker associated with transformation of prostate cells and tumorigenesis.

We observed that PSCA protein and mRNA are highly expressed in a large percentage of human prostate cancers, including advanced, poorly differentiated, androgen-independent and metastatic cases. Fluorescence-activated cell sorting and confocal/ immunofluorescent studies demonstrated cell surface expression of PSCA protein in Pca cells [9]. Our IHC expression analysis of PSCA shows not only cell surface but also apparent cytoplasmic staining of PSCA protein in Pca specimens (Figure 1). One possible explanation for this is that anti-PSCA antibody can recognize PSCA peptide precursors that reside in the cytoplasm. Also, it is possible that the positive staining that appears in the cytoplasm is actually from the overlying cell membrane [5]. These data seem to indicate that PSCA is a novel cell surface marker for human Pca.

Our results show that elevated level of PSCA expression correlates with high grade (i.e. poor differentiation), increased tumor stage and progression to androgen-independence of Pca. These findings support the original IHC analyses by Gu Z et al [9], who reported that PSCA protein expressed in 94% of primary Pca and the intensity of PSCA protein expression increased with tumor grade, stage and progression to androgen-independence. Our results also collaborate the recent work of Han KR et al [10], in which the significant association between high PSCA expression and adverse prognostic features such as high Gleason score, seminal vesicle invasion and capsular involvement in Pca was found. It is suggested that PSCA overexpression may be an adverse predictor for recurrence, clinical progression or survival of Pca. Hara H et al [11] used RT-PCR detection of PSA, PSMA and PSCA in 1

ml of peripheral blood to evaluate Pca patients with poor prognosis. The results showed that among 58 PCa patients, each PCR indicated the prognostic value in the hierarchy of PSCA>PSA>PSMA RT-PCR, and extraprostatic cases with positive PSCA PCR indicated lower disease-progression-free survival than those with negative PSCA PCR, demonstrating that PSCA can be used as a prognostic factor. Dubey P et al [12] reported that elevated numbers of PSCA + cells correlate positively with the onset and development of prostate carcinoma over a long time span in the prostates of the TRAMP and PTEN +/- models compared with its normal prostates. Taken together with our present findings, in which PSCA is overexpressed from HGPIN to almost frank carcinoma, it is reasonable and possible to use increased PSCA expression level or increased numbers of PSCA-positive cells in the prostate samples as a prognostic marker to predict the potential onset of this cancer. These data raise the possibility that PSCA may have diagnostic utility or clinical prognostic value in human Pca.

The cause of PSCA overexpression in Pca is not known. One possible mechanism is that it may result from PSCA gene amplification. In humans, PSCA is located on chromosome 8q24.2 [1], which is often amplified in metastatic and recurrent Pca and considered to indicate a poor prognosis [13-15]. Interestingly, PSCA is in close proximity to the c-myc oncogene, which is amplified in >20% of recurrent and metastatic prostate cancers [16,17]. Reiter RE et al [18] reported that PSCA and MYC gene copy numbers were co-amplified in 25% of tumors (five out of twenty), demonstrating that PSCA overexpression is associated with PSCA and MYC coamplification in Pca. Gu Z et al [9] recently reporteted that in 102 specimens available to compare the results of PSCA immunostaining with their previous mRNA ISH analysis, 92 (90.2%) had identically positive areas of PSCA protein and mRNA expression. Taken together with our findings, in which we detected moderate to strong expression of PSCA protein and mRNA in 34 of 40 (85%) Pca specimens examined simultaneously by IHC and ISH analyses, it is demonstrated that PSCA protein and mRNA overexpressed in human Pca, and that the increased protein level of PSCA was resulted from the upregulated transcription of its mRNA.

At present, the regulation mechanisms of human PSCA expression and its biological function are yet to be elucidated. PSCA expression may be regulated by multiple factors [18]. Watabe T et al [19] reported that transcriptional control is a major component regulating PSCA expression levels. In addition, induction of PSCA expression may be regulated or mediated through cell-cell contact and protein kinase C (PKC) [20]. Homologues of PSCA have diverse activities, and have themselves been involved in

carcinogenesis. Signalling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes [21]. Thy-1 is involved in T cell activation and transducts signals through src-like tyrosine kinases [22]. Ly-6 genes have been implicated both in tumorigenesis and in cell-cell adhesion [23-25]. Cell-cell or cell-matrix interaction is critical for local tumor growth and spread to distal sites. From its restricted expression in basal cells of normal prostate and its homology to SCA-2, PSCA may play a role in stem/progenitor cell function, such as self-renewal (i.e. anti-apoptosis) and/or proliferation [1]. Taken together with the results in the present study, we speculate that PSCA may play a role in tumorigenesis and clinical progression of Pca through affecting cell transformation and proliferation. From our results, it is also suggested that PSCA as a new cell surface antigen may have a number of potential uses in the diagnosis, therapy and clinical prognosis of human Pca. PSCA overexpression in prostate biopsies could be used to identify patients at high risk to develop recurrent or metastatic disease, and to discriminate cancers from normal glands in prostatectomy samples. Similarly, the detection of PSCA-overexpressing cells in bone marrow or peripheral blood may identify and predict metastatic progression better than current assays, which identify only PSA-positive or PSMA-positive prostate cells.

In summary, we have shown in this study that PSCA protein and mRNA are maintained in expression from HGPIN through all stages of Pca in a majority of cases, which may be associated with prostate carcinogenesis and correlate positively with high tumor grade (poor cell differentiation), advanced stage and androgen-independent progression. PSCA protein overexpression is due to the upregulation of its mRNA transcription. The results suggest that PSCA may be a promising molecular marker for the clinical prognosis of human Pca and a valuable target for diagnosis and therapy of this tumor.

Competing interests

None declared.

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Exhibit 8

Review

Translation Initiation in Cancer: A Novel Target for Therapy[1]

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Abstract

Translation initiation is regulated in response to nutrient availability and mitogenic stimulation and is coupled with cell cycle progression and cell growth. Several alterations in translational control occur in cancer. Variant mRNA sequences can alter the translational efficiency of individual mRNA molecules, which in turn play a role in cancer biology. Changes in the expression or availability of components of the translational machinery and in the activation of translation through signal transduction pathways can lead to more global changes, such as an increase in the overall rate of protein synthesis and translational activation of the mRNA molecules involved in cell growth and proliferation. We review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to help elucidate new therapeutic avenues.

Introduction

The fundamental principle of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells. With the advent of cDNA array technology, most efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable either to DNA amplification or to differences in transcription. Gene expression is quite complicated, however, and is also regulated at the level of mRNA stability, mRNA translation, and protein stability.

The power of translational regulation has been best recognized among developmental biologists, because transcription does not occur in early embryogenesis in eukaryotes. For example, in *Xenopus*, the period of transcriptional quiescence continues until the embryo reaches midblastula transition, the 4000-cell stage. Therefore, all necessary mRNA molecules are transcribed during oogenesis and stockpiled in a translationally inactive, masked form. The mRNA are translationally activated at appropriate times during oocyte maturation, fertilization, and early embryogenesis and thus, are under strict translational control.

Translation has an established role in cell growth. Basically, an increase in protein synthesis occurs as a consequence of mitogenesis. Until recently, however, little was known about the alterations in mRNA translation in cancer, and much is yet to be discovered about their role in the development and progression of cancer. Here we review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to elucidate potential new therapeutic avenues.

Basic Principles of Translational Control
Mechanism of Translation Initiation

Translation initiation is the main step in translational regulation. Translation initiation is a complex process in which the initiator tRNA and the 40S and 60S ribosomal subunits are recruited to the 5′ end of a mRNA molecule and assembled by eukaryotic translation initiation factors into an 80S ribosome at the start codon of the mRNA (Fig. 1). The 5′ end of eukaryotic mRNA is capped, i.e., contains the cap structure m^{7}GpppN (7-methyl-guanosine-triphospho-5′-ribonucleoside). Most translation in eukaryotes occurs in a cap-dependent fashion, i.e., the cap is specifically recognized by the eIF4E,[3] which binds the 5′ cap. The eIF4F translation initiation complex is then formed by the assembly of eIF4E, the RNA helicase eIF4A, and eIF4G, a scaffolding protein that mediates the binding of the 40S ribosomal subunit to the mRNA molecule through interaction with the eIF3 protein present on the 40S ribosome. eIF4A and eIF4B participate in melting the secondary structure of the 5′ UTR of the mRNA. The 43S initiation complex (40S/eIF2/Met-tRNA/GTP complex) scans the mRNA in a 5′→3′ direction until it encounters an AUG start codon. This start codon is then base-paired to the anticodon of initiator tRNA, forming the 48S initiation complex. The initiation factors are then displaced from the 48S complex, and the 60S ribosome joins to form the 80S ribosome.

Unlike most eukaryotic translation, translation initiation of certain mRNAs, such as the picornavirus RNA, is cap independent and occurs by internal ribosome entry. This mechanism does not require eIF4E. Either the 43S complex can bind the initiation codon directly through interaction with the IRES in the 5′ UTR such as in the encephalomyocarditis virus, or it can

[3] The abbreviations used are: eIF4E, eukaryotic initiation factor 4E; UTR, untranslated region; IRES, internal ribosome entry site; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K, ribosomal p70 S6 kinase; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PP2A, protein phosphatase 2A; TGF-β3, transforming growth factor-β3; PAP, poly(A) polymerase; EPA, eicosopentaenoic acid; mda-7, melanoma differentiation-associated gene 7.
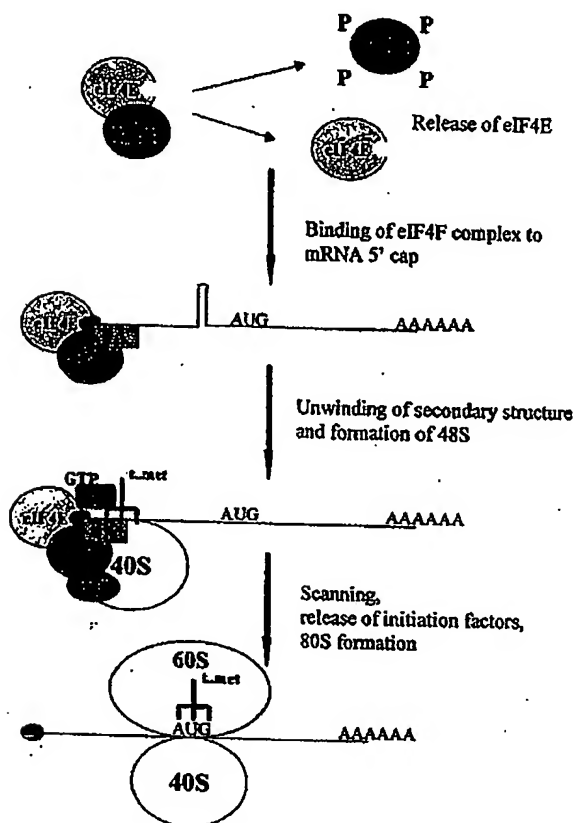
Fig. 1.   Translation initiation in eukaryotes. The 4E-BPs are hyperphosphorylated to release eIF4E so that it can interact with the 5' cap, and the eIF4F initiation complex is assembled. The interaction of poly(A) binding protein with the initiation complex and circularization of the mRNA is not depicted in the diagram. The secondary structure of the 5' UTR is melted, the 40S ribosomal subunit is bound to eIF3, and the ternary complex consisting of eIF2, GTP, and the Met-tRNA are recruited to the mRNA. The ribosome scans the mRNA in a 5'→3' direction until an AUG start codon is found in the appropriate sequence context. The initiation factors are released, and the large ribosomal subunit is recruited.

initially attach to the IRES and then reach the initiation codon by scanning or transfer, as is the case with the poliovirus (1).

Regulation of Translation Initiation

Translation Initiation can be regulated by alterations in the expression or phosphorylation status of the various factors involved. Key components in translational regulation that may provide potential therapeutic targets follow.

eIF4E. eIF4E plays a central role in translation regulation. It is the least abundant of the initiation factors and is considered the rate-limiting component for initiation of cap-dependent translation. eIF4E may also be involved in mRNA splicing, mRNA 3' processing, and mRNA nucleocytoplasmic transport (2). eIF4E expression can be increased at the transcriptional level in response to serum or growth factors (3). eIF4E overexpression may cause preferential translation of mRNAs containing excessive secondary structure in their 5' UTR that are normally discriminated against by the trans-

lational machinery and thus are inefficiently translated (4–7). As examples of this, overexpression of eIF4E promotes increased translation of vascular endothelial growth factor, fibroblast growth factor-2, and cyclin D1 (2, 8, 9).

Another mechanism of control is the regulation of eIF4E phosphorylation. eIF4E phosphorylation is mediated by the mitogen-activated protein kinase-interacting kinase 1, which is activated by the mitogen-activated pathway activating extracellular signal-related kinases and the stress-activated pathway acting through p38 mitogen-activated protein kinase (10–13). Several mitogens, such as serum, platelet-derived growth factor, epidermal growth factor, insulin, angiotensin II, src kinase overexpression, and ras overexpression, lead to eIF4E phosphorylation (14). The phosphorylation status of eIF4E is usually correlated with the translational rate and growth status of the cell; however, eIF4E phosphorylation has also been observed in response to some cellular stresses when translational rates actually decrease (15). Thus, further study is needed to understand the effects of eIF4E phosphorylation on eIF4E activity.

Another mechanism of regulation is the alteration of eIF4E availability by the binding of eIF4E to the eIF4E-binding proteins (4E-BP, also known as PHAS-I). 4E-BPs compete with eIF4G for a binding site in eIF4E. The binding of eIF4E to the best characterized eIF4E-binding protein, 4E-BP1, is regulated by 4E-BP1 phosphorylation. Hypophosphorylated 4E-BP1 binds to eIF4E, whereas 4E-BP1 hyperphosphorylation decreases this binding. Insulin, angiotensin, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, nerve growth factor, insulin-like growth factors I and II, interleukin 3, granulocyte-macrophage colony-stimulating factor + steel factor, gastrin, and the adenovirus have all been reported to induce phosphorylation of 4E-BP1 and to decrease the ability of 4E-BP1 to bind eIF4E (15, 16). Conversely, deprivation of nutrients or growth factors results in 4E-BP1 dephosphorylation, an increase in eIF4E binding, and a decrease in cap-dependent translation.

p70 S6 Kinase. Phosphorylation of ribosomal 40S protein S6 by S6K is thought to play an important role in translational regulation. S6K −/− mouse embryonic cells proliferate more slowly than do parental cells, demonstrating that S6K has a positive influence on cell proliferation (17). S6K regulates the translation of a group of mRNAs possessing a 5' terminal oligopyrimidine tract (5' TOP) found at the 5' UTR of ribosomal protein mRNAs and other mRNAs coding for components of the translational machinery. Phosphorylation of S6K is regulated in part based on the availability of nutrients (18, 19) and is stimulated by several growth factors, such as platelet-derived growth factor and insulin-like growth factor I (20).

eIF2α Phosphorylation. The binding of the initiator tRNA to the small ribosomal unit is mediated by translation initiation factor eIF2. Phosphorylation of the α-subunit of eIF2 prevents formation of the eIF2/GTP/Met-tRNA complex and inhibits global protein synthesis (21, 22). eIF2α is phosphorylated under a variety of conditions, such as viral infection, nutrient deprivation, heme deprivation, and apoptosis (22). eIF2α is phosphorylated by heme-regulated inhibitor, nutrient-regulated protein kinase, and the IFN-induced, double-stranded RNA-activated protein kinase (PKR; Ref. 23).

The mTOR Signaling Pathway. The macrolide antibiotic rapamycin (Sirolimus; Wyeth-Ayerst Research, Collegeville, PA) has been the subject of intensive study because it inhibits signal transduction pathways involved in T-cell activation. The rapamycin-sensitive component of these pathways is mTOR (also called FRAP or RAFT1). mTOR is the mammalian homologue of the yeast TOR proteins that regulate G_1 progression and translation in response to nutrient availability (24). mTOR is a serine-threonine kinase that modulates translation initiation by altering the phosphorylation status of 4E-BP1 and S6K (Fig. 2; Ref. 25).

4E-BP1 is phosphorylated on multiple residues. mTOR phosphorylates the Thr-37 and Thr-46 residues of 4E-BP1 in vitro (26); however, phosphorylation at these sites is not associated with a loss of eIF4E binding. Phosphorylation of Thr-37 and Thr-46 is required for subsequent phosphorylation at several COOH-terminal, serum-sensitive sites; a combination of these phosphorylation events appears to be needed to inhibit the binding of 4E-BP1 to eIF4E (25). The product of the ATM gene, p38/MSK1 pathway, and protein kinase Cσ also play a role in 4E-BP1 phosphorylation (27-29).

S6K and 4E-BP1 are also regulated, in part, by PI3K and its downstream protein kinase Akt. PTEN is a phosphatase that negatively regulates PI3K signaling. PTEN null cells have constitutively active of Akt, with increased S6K activity and S6 phosphorylation (30). S6K activity is inhibited both by PI3K inhibitors wortmannin and LY294002 and by mTOR inhibitor rapamycin (24). Akt phosphorylates Ser-2448 in mTOR in vitro, and this site is phosphorylated upon Akt activation in vivo (31-33). Thus, mTOR is regulated by the PI3K/Akt pathway; however, this does not appear to be the only mode of regulation of mTOR activity. Whether the PI3K pathway also regulates S6K and 4E-BP1 phosphorylation independent of mTOR is controversial.

Interestingly, mTOR autophosphorylation is blocked by wortmannin but not by rapamycin (34). This seeming inconsistency suggests that mTOR-responsive regulation of 4E-BP1 and S6K activity occurs through a mechanism other than intrinsic mTOR kinase activity. An alternate pathway for 4E-BP1 and S6K phosphorylation by mTOR activity is by the inhibition of a phosphatase. Treatment with calyculin A, an inhibitor of phosphatases 1 and 2A, reduces rapamycin-induced dephosphorylation of 4E-BP1 and S6K by rapamycin (35). PP2A interacts with full-length S6K but not with a S6K mutant that is resistant to dephosphorylation resulting from rapamycin. mTOR phosphorylates PP2A in vitro; however, how this process alters PP2A activity is not known. These results are consistent with the model that phosphorylation of a phosphatase by mTOR prevents dephosphorylation of 4E-BP1 and S6K, and conversely, that nutrient deprivation and rapamycin block inhibition of the phosphatase by mTOR.

Polyadenylation. The poly(A) tail in eukaryotic mRNA is important in enhancing translation initiation and mRNA stability. Polyadenylation plays a key role in regulating gene expression during oogenesis and early embryogenesis. Some mRNA that are translationally inactive in the oocyte are polyadenylated concomitantly with translational activation in oocyte maturation, whereas other mRNAs that are translationally active during oogenesis are deadenylated and trans-
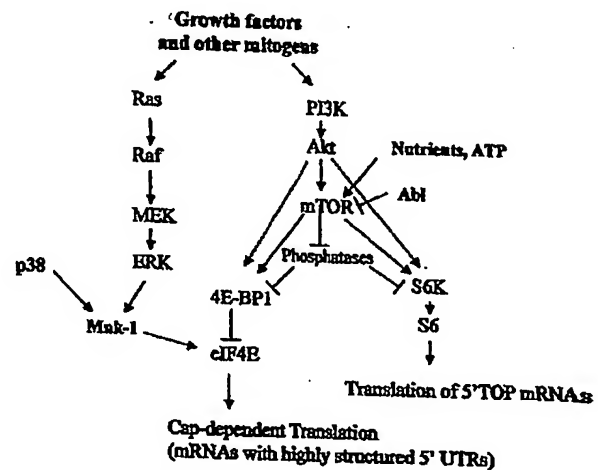

Fig. 2. Regulation of translation initiation by signal transduction pathways. Signaling via p38, extracellular signal-related kinase, PI3K, and mTOR can all activate translation initiation.

lationally silenced (36-38). Thus, control of poly(A) tail synthesis is an important regulatory step in gene expression. The 5' cap and poly(A) tail are thought to function synergistically to regulate mRNA translational efficiency (39, 40).

RNA Packaging. Most RNA-binding proteins are assembled on a transcript at the time of transcription, thus determining the translational fate of the transcript (41). A highly conserved family of Y-box proteins is found in cytoplasmic messenger ribonucleoprotein particles, where the proteins are thought to play a role in restricting the recruitment of mRNA to the translational machinery (41-43). The major mRNA-associated protein, YB-1, destabilizes the interaction of eIF4E and the 5' mRNA cap in vitro, and overexpression of YB-1 results in translational repression in vivo (44). Thus, alterations in RNA packaging can also play an important role in translational regulation.

Translation Alterations Encountered in Cancer

Three main alterations at the translational level occur in cancer: variations in mRNA sequences that increase or decrease translational efficiency, changes in the expression or availability of components of the translational machinery, and activation of translation through aberrantly activated signal transduction pathways. The first alteration affects the translation of an individual mRNA that may play a role in carcinogenesis. The second and third alterations can lead to more global changes, such as an increase in the overall rate of protein synthesis, and the translational activation of several mRNA species.

Variations in mRNA Sequence

Variations in mRNA sequence affect the translational efficiency of the transcript. A brief description of these variations and examples of each mechanism follow.

Mutations. Mutations in the mRNA sequence, especially in the 5' UTR, can alter its translational efficiency, as seen in the following examples.

c-myc. Saito et al. proposed that translation of full-length c-myc is repressed, whereas in several Burkitt lymphomas that have deletions of the mRNA 5' UTR, translation of c-myc is more efficient (45). More recently, it was reported that the 5' UTR of c-myc contains an IRES, and thus c-myc translation can be initiated by a cap-independent as well as a cap-dependent mechanism (46, 47). In patients with multiple myeloma, a C→T mutation in the c-myc IRES was identified (48) and found to cause an enhanced initiation of translation via internal ribosomal entry (49).

BRCA1. A somatic point mutation (117 G→C) in position −3 with respect to the start codon of the BRCA1 gene was identified in a highly aggressive sporadic breast cancer (50). Chimeric constructs consisting of the wild-type or mutated BRCA1 5' UTR and a downstream luciferase reporter demonstrated a decrease in the translational efficiency with the 5' UTR mutation.

Cyclin-dependent Kinase Inhibitor 2A. Some inherited melanoma kindreds have a G→T transversion at base −34 of cyclin-dependent kinase inhibitor-2A, which encodes a cyclin-dependent kinase 4/cyclin-dependent kinase 6 kinase inhibitor important in G_1 checkpoint regulation (51). This mutation gives rise to a novel AUG translation initiation codon, creating an upstream open reading frame that competes for scanning ribosomes and decreases translation from the wild-type AUG.

Alternate Splicing and Alternate Transcription Start Sites. Alterations in splicing and alternate transcription sites can lead to variations in 5' UTR sequence, length, and secondary structure, ultimately impacting translational efficiency.

ATM. The ATM gene has four noncoding exons in its 5' UTR that undergo extensive alternative splicing (52). The contents of 12 different 5' UTRs that show considerable diversity in length and sequence have been identified. These divergent 5' leader sequences play an important role in the translational regulation of the ATM gene.

mdm. In a subset of tumors, overexpression of the oncoprotein mdm2 results in enhanced translation of the mdm2 mRNA. Use of different promoters leads to two mdm2 transcripts that differ only in their 5' leaders (53). The longer 5' UTR contains two upstream open reading frames, and this mRNA is loaded with ribosomes inefficiently compared with the short 5' UTR.

BRCA1. In a normal mammary gland, BRCA1 mRNA is expressed with a shorter leader sequence (5'UTRa), whereas in sporadic breast cancer tissue, BRCA1 mRNA is expressed with a longer leader sequence (5' UTRb); the translational efficiency of transcripts containing 5' UTRb is 10 times lower than that of transcripts containing 5' UTRa (54).

TGF-β3. TGF-β3 mRNA includes a 1.1-kb 5' UTR, which exerts an inhibitory effect on translation. Many human breast cancer cell lines contain a novel TGF-β3 transcript with a 5' UTR that is 870 nucleotides shorter and has a 7-fold greater translational efficiency than the normal TGF-β3 mRNA (55).

Alternate Polyadenylation Sites. Multiple polyadenylation signals leading to the generation of several transcripts with differing 3' UTR have been described for several mRNA species, such as the RET proto-oncogene (56), ATM gene (52), tissue inhibitor of metalloproteinases-3 (57), RHOA

proto-oncogene (58), and calmodulin-I (59). Although the effect of these alternate 3' UTRs on translation is not yet known, they may be important in RNA-protein interactions that affect translational recruitment. The role of these alterations in cancer development and progression is unknown.

Alterations in the Components of the Translation Machinery

Alterations in the components of translation machinery can take many forms.

Overexpression of eIF4E. Overexpression of eIF4E causes malignant transformation in rodent cells (60) and the deregulation of HeLa cell growth (61). Polunovsky et al. (62) found that eIF4E overexpression substitutes for serum and individual growth factors in preserving viability of fibroblasts, which suggests that eIF4E can mediate both proliferative and survival signaling.

Elevated levels of eIF4E mRNA have been found in a broad spectrum of transformed cell lines (63). eIF4E levels are elevated in all ductal carcinoma in situ specimens and invasive ductal carcinomas, compared with benign breast specimens evaluated with Western blot analysis (64, 65). Preliminary studies suggest that this overexpression is attributable to gene amplification (66).

There are accumulating data suggesting that eIF4E overexpression can be valuable as a prognostic marker. eIF4E overexpression was found in a retrospective study to be a marker of poor prognosis in stages I to III breast carcinoma (67). Verification of the prognostic value of eIF4E in breast cancer is now under way in a prospective trial (67). However, in a different study, eIF4E expression was correlated with the aggressive behavior of non-Hodgkin's lymphomas (68). In a prospective analysis of patients with head and neck cancer, elevated levels of eIF4E in histologically tumor-free surgical margins predicted a significantly increased risk of local-regional recurrence (9). These results all suggest that eIF4E overexpression can be used to select patients who might benefit from more aggressive systemic therapy. Furthermore, the head and neck cancer data suggest that eIF4E overexpression is a field defect and can be used to guide local therapy.

Alterations in Other Initiation Factors. Alterations in a number of other initiation factors have been associated with cancer. Overproduction of eIF4G, similar to eIF4E, leads to malignant transformation in vitro (69). eIF-2α is found in increased levels in bronchioloalveolar carcinomas of the lung (3). Initiation factor eIF-4A1 is overexpressed in melanoma (70) and hepatocellular carcinoma (71). The p40 subunit of translation initiation factor 3 is amplified and overexpressed in breast and prostate cancer (72), and the eIF3-p110 subunit is overexpressed in testicular seminoma (73). The role that overexpression of these initiation factors plays on the development and progression of cancer, if any, is not known.

Overexpression of S6K. S6K is amplified and highly overexpressed in the MCF7 breast cancer cell line, compared with normal mammary epithelium (74). In a study by Barlund et al. (74), S6K was amplified in 59 of 668 primary breast tumors, and a statistically significant association was observed between amplification and poor prognosis.

Overexpression of PAP. PAP catalyzes 3′ poly(A) synthesis. PAP is overexpressed in human cancer cells compared with normal and virally transformed cells (75). PAP enzymatic activity in breast tumors has been correlated with PAP protein levels (76) and, in mammary tumor cytosols, was found to be an independent factor for predicting survival (76). Little is known, however, about how PAP expression or activity affects the translational profile.

Alterations in RNA-binding Proteins. Even less is known about alterations in RNA packaging in cancer. Increased expression and nuclear localization of the RNA-binding protein YB-1 are indicators of a poor prognosis for breast cancer (77), non-small cell lung cancer (78), and ovarian cancer (79). However, this effect may be mediated at least in part at the level of transcription, because YB-1 increases chemoresistance by enhancing the transcription of a multidrug resistance gene (80).

Activation of Signal Transduction Pathways

Activation of signal transduction pathways by loss of tumor suppressor genes or overexpression of certain tyrosine kinases can contribute to the growth and aggressiveness of tumors. An important mutant in human cancers is the tumor suppressor gene *PTEN*, which leads to the activation of the PI3K/Akt pathway. Activation of PI3K and Akt induces the oncogenic transformation of chicken embryo fibroblasts. The transformed cells show constitutive phosphorylation of S6K and of 4E-BP1 (81). A mutant Akt that retains kinase activity but does not phosphorylate S6K or 4E-BP1 does not transform fibroblasts, which suggests a correlation between the oncogenicity of PI3K and Akt and the phosphorylation of S6K and 4E-BP1 (81).

Several tyrosine kinases such as platelet-derived growth factor, insulin-like growth factor, HER2/neu, and epidermal growth factor receptor are overexpressed in cancer. Because these kinases activate downstream signal transduction pathways known to alter translation initiation, activation of translation is likely to contribute to the growth and aggressiveness of these tumors. Furthermore, the mRNA for many of these kinases themselves are under translational control. For example, HER2/neu mRNA is translationally controlled both by a short upstream open reading frame that represses HER2/neu translation in a cell type-independent manner and by a distinct cell type-dependent mechanism that increases translational efficiency (82). HER2/neu translation is different in transformed and normal cells. Thus, it is possible that alterations at the translational level can in part account for the discrepancy between *HER2/neu* gene amplification detected by fluorescence *in situ* hybridization and protein levels detected by immunohistochemical assays.

Translation Targets of Selected Cancer Therapy

Components of the translation machinery and signal pathways involved in the activation of translation initiation represent good targets for cancer therapy.

Targeting the mTOR Signaling Pathway: Rapamycin and Tumstatin

Rapamycin inhibits the proliferation of lymphocytes. It was initially developed as an immunosuppressive drug for organ transplantation. Rapamycin with FKBP 12 (FK508-binding protein, M_r 12,000) binds to mTOR to inhibit its function.

Rapamycin causes a small but significant reduction in the initiation rate of protein synthesis (83). It blocks cell growth in part by blocking S6 phosphorylation and selectively suppressing the translation of 5′ TOP mRNAs, such as ribosomal proteins, and elongation factors (83–85). Rapamycin also blocks 4E-BP1 phosphorylation and inhibits cap-dependent but not cap-independent translation (17, 86).

The rapamycin-sensitive signal transduction pathway, activated during malignant transformation and cancer progression, is now being studied as a target for cancer therapy (87). Prostate, breast, small cell lung, glioblastoma, melanoma, and T-cell leukemia are among the cancer lines most sensitive to the rapamycin analogue CCI-779 (Wyeth-Ayerst Research; Ref. 87). In rhabdomyosarcoma cell lines, rapamycin is either cytostatic or cytocidal, depending on the p53 status of the cell; p53 wild-type cells treated with rapamycin arrest in the G_1 phase and maintain their viability, whereas p53 mutant cells accumulate in G_1 and undergo apoptosis (88, 89). In a recently reported study using human primitive neuroectodermal tumor and medulloblastoma models, rapamycin exhibited more cytotoxicity in combination with cisplatin and camptothecin than as a single agent. In vivo, CCI-779 delayed growth of xenografts by 160% after 1 week of therapy and 240% after 2 weeks. A single high-dose administration caused a 37% decrease in tumor volume. Growth inhibition in vivo was 1.3 times greater, with cisplatin in combination with CCI-779 than with cisplatin alone (90). Thus, preclinical studies suggest that rapamycin analogues are useful as single agents and in combination with chemotherapy.

Rapamycin analogues CCI-779 and RAD001 (Novartis, Basel, Switzerland) are now in clinical trials. Because of the known effect of rapamycin on lymphocyte proliferation, a potential problem with rapamycin analogues is immunosuppression. However, although prolonged immunosuppression can result from rapamycin and CCI-779 administered on continuous-dose schedules, the immunosuppressive effects of rapamycin analogues resolve in ~24 h after therapy (91). The principal toxicities of CCI-779 have included dermatological toxicity, myelosuppression, infection, mucositis, diarrhea, reversible elevations in liver function tests, hyperglycemia, hypokalemia, hypocalcemia, and depression (87, 92–94). Phase II trials of CCI-779 have been conducted in advanced renal cell carcinoma and in stage III/IV breast carcinoma patients who failed with prior chemotherapy. In the results reported in abstract form, although there were no complete responses, partial responses were documented in both renal cell carcinoma and in breast carcinoma (94, 95). Thus, CCI-779 has documented preliminary clinical activity in a previously treated, unselected patient population.

Active investigation is under way into patient selection for mTOR inhibitors. Several studies have found an enhanced efficacy of CCI-779 in PTEN-null tumors (30, 96). Another study found that six of eight breast cancer cell lines were responsive to CCI-779, although only two of these lines lacked PTEN (97) There was, however, a positive correlation between Akt activation and CCI-779 sensitivity (97). This correlation suggests that activation of the PI3K-Akt pathway,

regardless of whether it is attributable to a PTEN mutation or to overexpression of receptor tyrosine kinases, makes cancer cell amenable to mTOR-directed therapy. In contrast, lower levels of the target of mTOR, 4E-BP1, are associated with rapamycin resistance; thus, a lower 4E-BP1/eIF4E ratio may predict rapamycin resistance (98).

Another mode of activity for rapamycin and its analogues appears to be through inhibition of angiogenesis. This activity may be both through direct inhibition of endothelial cell proliferation as a result of mTOR inhibition in these cells or by inhibition of translation of such proangiogenic factors as vascular endothelial growth factor in tumor cells (99, 100).

The angiogenesis inhibitor tumstatin, another anticancer drug currently under study, was also found recently to inhibit translation in endothelial cells (101). Through a requisite interaction with integrin, tumstatin inhibits activation of the PI3K/Akt pathway and mTOR in endothelial cells and prevents dissociation of eIF4E from 4E-BP1, thereby inhibiting cap-dependent translation. These findings suggest that endothelial cells are especially sensitive to therapies targeting the mTOR-signaling pathway.

Targeting eIF2α: EPA, Clotrimazole, mda-7, and Flavonoids

EPA is an n-3 polyunsaturated fatty acid found in the fish-based diets of populations having a low incidence of cancer (102). EPA inhibits the proliferation of cancer cells (103), as well as in animal models (104, 105). It blocks cell division by inhibiting translation initiation (105). EPA releases Ca^{2+} from intracellular stores while inhibiting their refilling, thereby activating PKR. PKR, in turn phosphorylates and inhibits eIF2α, resulting in the inhibition of protein synthesis at the level of translation initiation. Similarly, clotrimazole, a potent antiproliferative agent in vitro and in vivo, inhibits cell growth through depletion of Ca^{2+} stores, activation of PKR, and phosphorylation of eIF2α (106). Consequently, clotrimazole preferentially decreases the expression of cyclins A, E, and D1, resulting in blockage of the cell cycle in G_1.

mda-7 is a novel tumor suppressor gene being developed as a gene therapy agent. Adenoviral transfer of mda-7 (Ad-mda7) induces apoptosis in many cancer cells including breast, colorectal, and lung cancer (107–109). Ad-mda7 also induces and activates PKR, which leads to phosphorylation of eIF2α and induction of apoptosis (110).

Flavonoids such as genistein and quercetin suppress tumor cell growth. All three mammalian eIF2α kinases, PKR, heme-regulated inhibitor, and PERK/PEK, are activated by flavonoids, with phosphorylation of eIF2α and inhibition of protein synthesis (111).

Targeting eIF4A and eIF4E: Antisense RNA and Peptides

Antisense expression of eIF4A decreases the proliferation rate of melanoma cells (112). Sequestration of eIF4E by overexpression of 4E-BP1 is proapoptotic and decreases tumorigenicity (113, 114). Reduction of eIF4E with antisense RNA decreases soft agar growth, increases tumor latency, and increases the rates of tumor doubling times (7). Antisense eIF4E RNA treat-

ment also reduces the expression of angiogenic factors (115) and has been proposed as a potential adjuvant therapy for head and neck cancers, particularly when elevated eIF4E is found in surgical margins. Small molecule inhibitors that bind the eIF4G/4E-BP1-binding domain of eIF4E are proapoptotic (116) and are also being actively pursued.

Exploiting Selective Translation for Gene Therapy

A different therapeutic approach that takes advantage of the enhanced cap-dependent translation in cancer cells is the use of gene therapy vectors encoding suicide genes with highly structured 5' UTR. These mRNA would thus be at a competitive disadvantage in normal cells and not translate well, whereas in cancer cells, they would translate more efficiently. For example, the introduction of the 5' UTR of fibroblast growth factor-2 5' to the coding sequence of herpes simplex virus type-1 thymidine kinase gene, allows for selective translation of herpes simplex virus type-1 thymidine kinase gene in breast cancer cell lines compared with normal mammary cell lines and results in selective sensitivity to ganciclovir (117).

Toward the Future

Translation is a crucial process in every cell. However, several alterations in translational control occur in cancer. Cancer cells appear to need an aberrantly activated translational state for survival, thus allowing the targeting of translation initiation with surprisingly low toxicity. Components of the translational machinery, such as eIF4E, and signal transduction pathways involved in translation initiation, such mTOR, represent promising targets for cancer therapy. Inhibitors of the mTOR have already shown some preliminary activity in clinical trials. It is possible that with the development of better predictive markers and better patient selection, response rates to single-agent therapy can be improved. Similar to other cytostatic agents, however, mTOR inhibitors are most likely to achieve clinical utility in combination therapy. In the interim, our increasing understanding of translation initiation and signal transduction pathways promise to lead to the identification of new therapeutic targets in the near future.

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Exhibit 9

Correlation between Protein and mRNA Abundance in Yeast

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We have determined the relationship between mRNA and protein expression levels for selected genes expressed in the yeast *Saccharomyces cerevisiae* growing at mid-log phase. The proteins contained in total yeast cell lysate were separated by high-resolution two-dimensional (2D) gel electrophoresis. Over 150 protein spots were excised and identified by capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein spots were quantified by metabolic labeling and scintillation counting. Corresponding mRNA levels were calculated from serial analysis of gene expression (SAGE) frequency tables (V. E. Velculescu, L. Zhang, W. Zhou, J. Vogelstein, M. A. Basrai, D. E. Bassett, Jr., P. Hieter, B. Vogelstein, and K. W. Kinzler, Cell 88:243–251, 1997). We found that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. Indeed, for some genes, while the mRNA levels were of the same value the protein levels varied by more than 20-fold. Conversely, invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that varied by as much as 30-fold. Another interesting observation is that codon bias is not a predictor of either protein or mRNA levels. Our results clearly delineate the technical boundaries of current approaches for quantitative analysis of protein expression and reveal that simple deduction from mRNA transcript analysis is insufficient.

The description of the state of a biological system by the quantitative measurement of the system constituents is an essential but largely unexplored area of biology. With recent technical advances including the development of differential display-PCR (21), of cDNA microarray and DNA chip technology (20, 27), and of serial analysis of gene expression (SAGE) (34, 35), it is now feasible to establish global and quantitative mRNA expression profiles of cells and tissues in species for which the sequence of all the genes is known. However, there is emerging evidence which suggests that mRNA expression patterns are necessary but are by themselves insufficient for the quantitative description of biological systems. This evidence includes discoveries of posttranscriptional mechanisms controlling the protein translation rate (15), the half-lives of specific proteins or mRNAs (33), and the intracellular location and molecular association of the protein products of expressed genes (32).

Proteome analysis, defined as the analysis of the protein complement expressed by a genome (26), has been suggested as an approach to the quantitative description of the state of a biological system by the quantitative analysis of protein expression profiles (36). Proteome analysis is conceptually attractive because of its potential to determine properties of biological systems that are not apparent by DNA or mRNA sequence analysis alone. Such properties include the quantity of protein expression, the subcellular location, the state of modification, and the association with ligands, as well as the rate of change with time of such properties. In contrast to the genomes of a number of microorganisms (for a review, see reference 11) and the transcriptome of *Saccharomyces cerevisiae* (35), which have been entirely determined, no proteome map has been completed to date.

The most common implementation of proteome analysis is the combination of two-dimensional gel electrophoresis (2DE)

(isoelectric focusing-sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis) for the separation and quantitation of proteins with analytical methods for their identification. 2DE permits the separation, visualization, and quantitation of thousands of proteins reproducibly on a single gel (18, 24). By itself, 2DE is strictly a descriptive technique. The combination of 2DE with protein analytical techniques has added the possibility of establishing the identities of separated proteins (1, 2) and thus, in combination with quantitative mRNA analysis, of correlating quantitative protein and mRNA expression measurements of selected genes.

The recent introduction of mass spectrometric protein analysis techniques has dramatically enhanced the throughput and sensitivity of protein identification to a level which now permits the large-scale analysis of proteins separated by 2DE. The techniques have reached a level of sensitivity that permits the identification of essentially any protein that is detectable in the gels by conventional protein staining (9, 29). Current protein analytical technology is based on the mass spectrometric generation of peptide fragment patterns that are idiotypic for the sequence of a protein. Protein identity is established by correlating such fragment patterns with sequence databases (10, 22, 37). Sophisticated computer software (8) has automated the entire process such that proteins are routinely identified with no human interpretation of peptide fragment patterns.

In this study, we have analyzed the mRNA and protein levels of a group of genes expressed in exponentially growing cells of the yeast *S. cerevisiae*. Protein expression levels were quantified by metabolic labeling of the yeast proteins to a steady state, followed by 2DE and liquid scintillation counting of the selected, separated protein species. Separated proteins were identified by in-gel tryptic digestion of spots with subsequent analysis by microspray liquid chromatography-tandem mass spectrometry (LC-MS/MS) and sequence database searching. The corresponding mRNA transcript levels were calculated from SAGE frequency tables (35).

This study, for the first time, explores a quantitative comparison of mRNA transcript and protein expression levels for a relatively large number of genes expressed in the same metabolic state. The resultant correlation is insufficient for predic-

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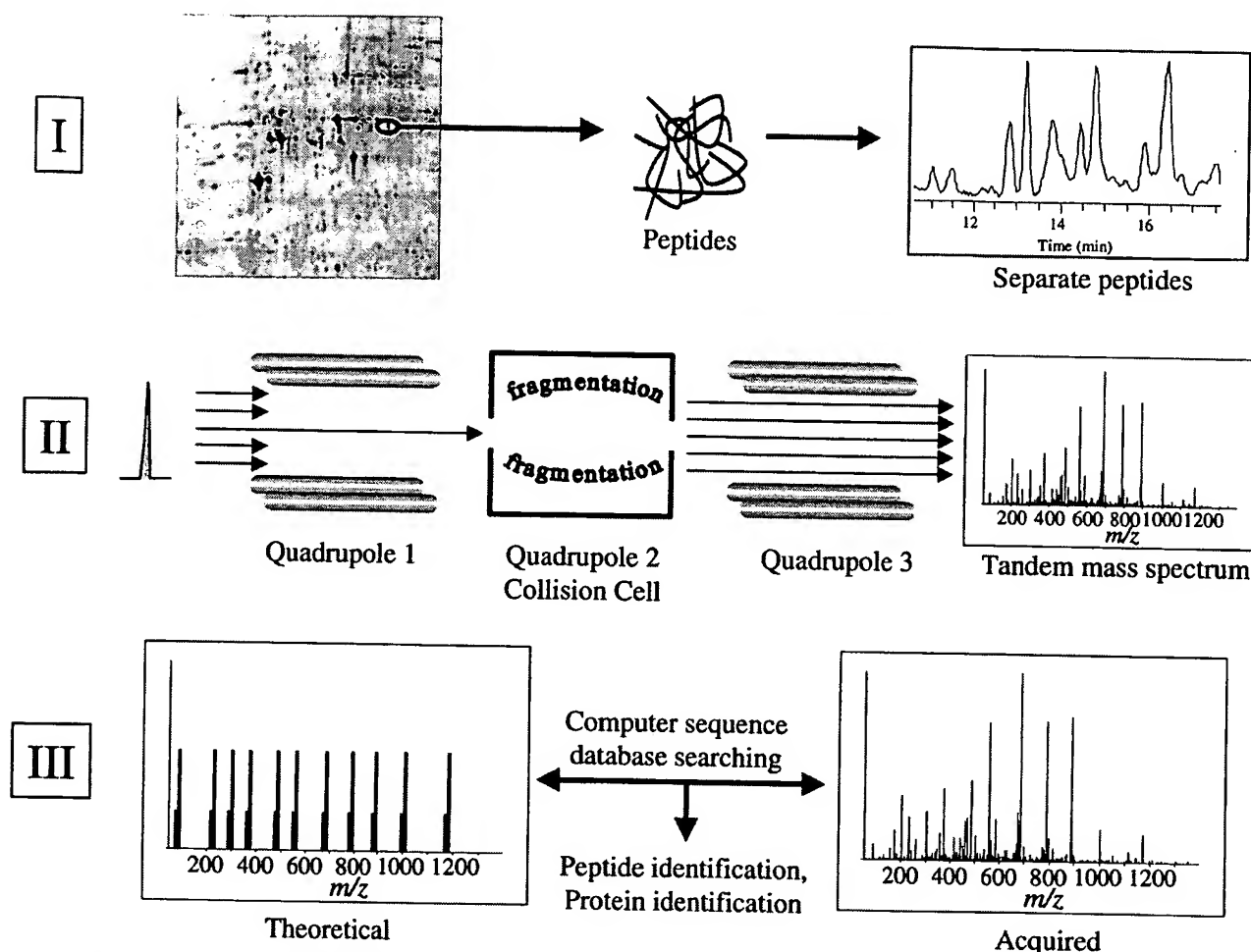
FIG. 1. Schematic illustration of proteome analysis by 2DE and mass spectrometry. In part I, proteins are separated by 2DE, stained spots are excised and subjected to in-gel digestion with trypsin, and the resulting peptides are separated by on-line capillary high-performance liquid chromatography. In part II, a peptide is shown eluting from the column in part I. The peptide is ionized by electrospray ionization and enters the mass spectrometer. The mass of the ionized peptide is detected, and the first quadrupole mass filter allows only the specific mass-to-charge ratio of the selected peptide ion to pass into the collision cell. In the collision cell, the energized, ionized peptides collide with neutral argon gas molecules. Fragmentation of the peptide is essentially random but occurs mainly at the peptide bonds, resulting in smaller peptides of differing lengths (masses). These peptide fragments are detected as a tandem mass (MS/MS) spectrum in the third quadrupole mass filter where two ion series are recorded simultaneously, one each from sequencing inward from the N and C termini of the peptide, respectively. In part III, the MS/MS spectrum from the selected, ionized peptide is compared to predicted tandem mass spectra computer generated from a sequence database. Provided that the peptide sequence exists in the database, the peptide and, by association, the protein from which the peptide was derived can be identified. Unambiguous protein identification is attained in a single analysis because multiple peptides are identified as being derived from the same protein.

tion of protein levels from mRNA transcript levels. We have also compared the relative amounts of protein and mRNA with the respective codon bias values for the corresponding genes. This comparison indicates that codon bias by itself is insufficient to accurately predict either the mRNA or the protein expression levels of a gene. In addition, the results demonstrate that only highly expressed proteins are detectable by 2DE separation of total cell lysates and that therefore the construction of complete proteome maps with current technology will be very challenging, irrespective of the type of organism.

MATERIALS AND METHODS

Yeast strain and growth conditions. The source of protein and message transcripts for all experiments was YPH499 (*MATa ura3-52 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 trp1-Δ63*) (30). Logarithmically growing cells were obtained by growing yeast cells to early log phase (3 × 10⁶ cells/ml) in YPD rich medium (YPD supplemented with 6 mM uracil, 4.8 mM adenine, and 24 mM tryptophan) at 30°C (35). Metabolic labeling of protein was accomplished in YPD medium

exactly as described elsewhere (4) with the exception that 1 ml of cells was labeled with 3 mCi to offset methionine present in YPD medium. Protein was harvested as described by Garrels and coworkers (12). Harvested protein was lyophilized, resuspended in isoelectric focusing gel rehydration solution, and stored at −80°C.

2DE. Soluble proteins were run in the first dimension by using a commercial flatbed electrophoresis system (Multiphor II; Pharmacia Biotech). Immobilized polyacrylamide gel (IPG) dry strips with nonlinear pH 3.0 to 10.0 gradients (Amersham-Pharmacia Biotech) were used for the first-dimension separation. Forty micrograms of protein from whole-cell lysates was mixed with IPG strip rehydration buffer (8 M urea, 2% Nonidet P-40, 10 mM dithiothreitol), and 250 to 380 μl of solution was added to individual lanes of an IPG strip rehydration tray (Amersham-Pharmacia Biotech). The strips were allowed to rehydrate at room temperature for 1 h. The samples were run at 300 V–10 mA–5 W for 2 h, then ramped to 3,500 V–10 mA–5 W over a period of 3 h, and then kept at 3,500 V–10 mA–5 W for 15 to 19 h. At the end of the first-dimension run (60 to 70 kV · h), the IPG strips were reequilibrated for 8 min in 2% (wt/vol) dithiothreitol in 2% (wt/vol) SDS–6 M urea–30% (wt/vol) glycerol–0.05 M Tris HCl (pH 6.8) and for 4 min in 2.5% iodoacetamide in 2% (wt/vol) SDS–6 M urea–30% (wt/vol) glycerol–0.05 M Tris HCl (pH 6.8). Following reequilibration, the strips were transferred and apposed to 10% polyacrylamide second-dimension gels. Poly-acrylamide gels were poured in a casting stand with 10% acrylamide–2.67% piperazine diacrylamide–0.375 M Tris base-HCl (pH 8.8)–0.1% (wt/vol) SDS–0.05%
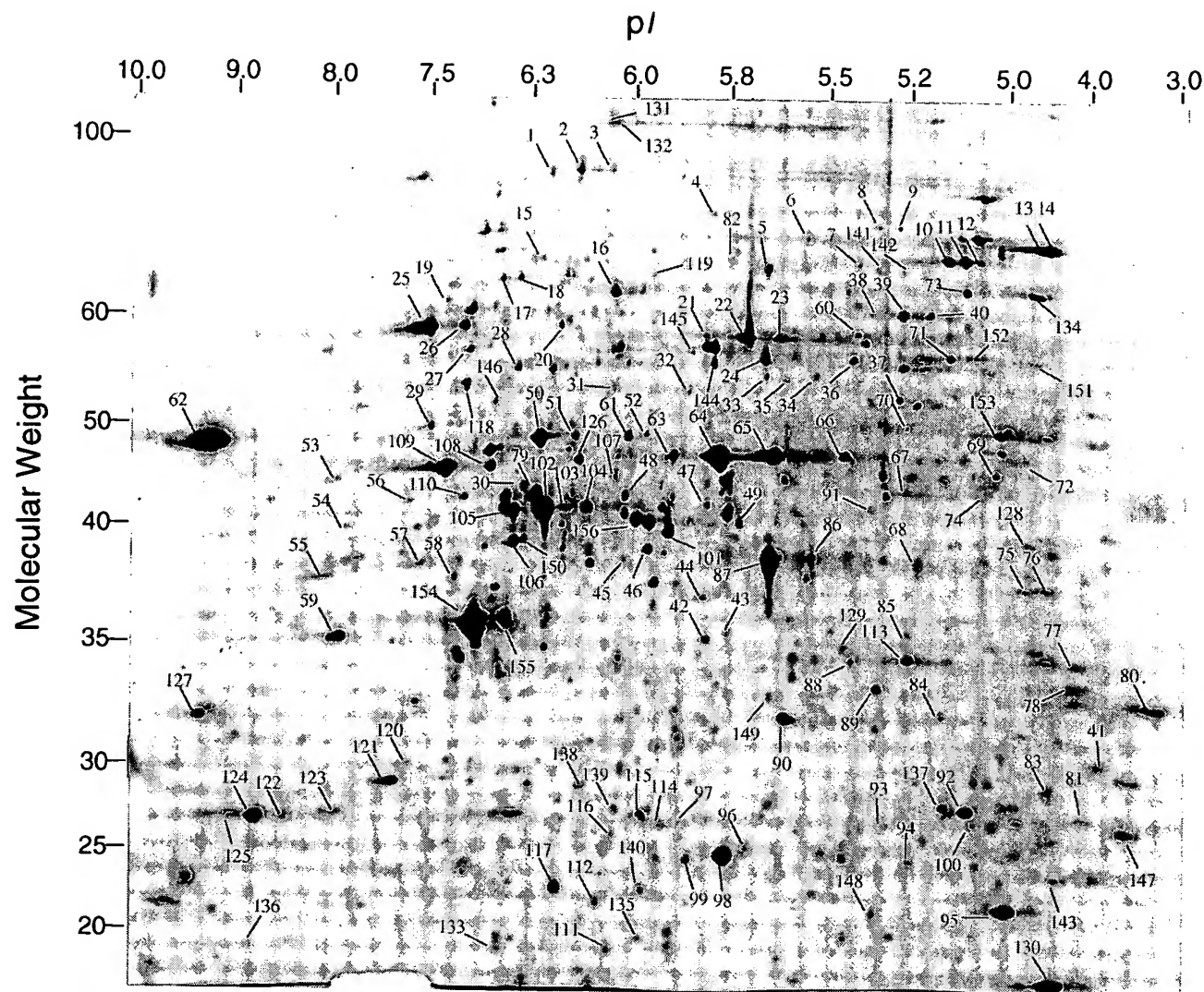
FIG. 2. 2D silver-stained gel of the proteins in yeast total cell lysate. Proteins were separated in the first dimension (horizontal) by isoelectric focusing and then in the second dimension (vertical) by molecular weight sieving. Protein spots (156) were chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities. Spots were excised, and the corresponding protein was identified by mass spectrometry and database searching. The spots are labeled on the gel and correspond to the data presented in Table 1. Molecular weights are given in thousands.

(wt/vol) ammonium persulfate–0.05% TEMED (N,N,N',N'-tetramethylethylenediamine) in Milli-Q water. The apparatus used to run second-dimension gels was a noncommercial apparatus from Oxford Glycosciences, Inc. Once the IPG strips were apposed to the second-dimension gels, they were immediately run at 50 mA (constant)–500 V–85 W for 20 min, followed by 200 mA (constant)–500 V–85 W until the buffer front line was 10 to 15 mm from the bottom of the gel. Gels were removed and silver stained according to the procedure of Shevchenko et al. (29).

Protein identification. Gels were exposed to X-ray film overnight, and then the silver staining and film were used to excise 156 spots of varying intensities, molecular weights, and isoelectric focusing points. In order to increase the detection limit by mass spectrometry, spots were cut out and pooled from up to four identical cold, silver-stained gels. In-gel tryptic digests of pooled spots were performed as described previously (29). Tryptic peptides were analyzed by microcapillary LC-MS with automated switching to MS/MS mode for peptide fragmentation. Spectra were searched against the composite OWL protein sequence database (version 30.2; 250,514 protein sequences) (24a) by using the computer program Sequest (8), which matches theoretical and acquired tandem mass spectra. A protein match was determined by comparing the number of peptides identified and their respective cross-correlation scores. All protein identifications were verified by comparison with theoretical molecular weights and isoelectric points.

mRNA quantitation. Velculescu and coworkers have previously generated frequency tables for yeast mRNA transcripts from the same strain grown under the same stated conditions as described herein (35). The SAGE technology is based on two main principles. First, a short sequence tag (15 bp) that contains sufficient information uniquely to identify a transcript is generated. A single tag is usually generated from each mRNA transcript in the cell which corresponds to 15 bp at the 3'-most cutting site for NlaIII. Second, many transcript tags can be concatenated into a single molecule and then sequenced, revealing the identity of multiple tags simultaneously. Over 20,000 transcripts were sequenced from yeast strain YPH499 growing at mid-log phase on glucose. Assuming the previously derived estimate of 15,000 mRNA molecules per cell (16), this would represent a 1.3-fold coverage even for mRNA molecules present at a single copy per cell and would provide a 72% probability of detecting such transcripts. Computer software which took for input the gene detected, examined the nucleotide sequence, and performed the calculation as described by Velculescu and coworkers (35) was written. In practice, we found that for 21 of 128 (16%) genes examined viable mRNA levels from SAGE data could not be calculated. This was because (i) no CATG site was found in the open reading frame (ORF), (ii) a CATG site was found but the corresponding 10-bp putative SAGE tag was not found in the frequency tables, or (iii) identical putative SAGE tags were present for multiple genes (e.g., TDH2_YEAST and TDH3_YEAST).

TABLE 1. Expressed genes identified from 2D gel in Fig. 2 — Continued

Mol wt	pI	Spot no.	YPD gene name[a]	Protein abundance (10³ copies/cell)	mRNA abundance (copies/cell)	Codon bias
17,259	6.75	133	CPR1	15.2	61.7	0.769
18,702	4.80	83	EGD2	20.1	5.2	0.724
18,726	4.44	147	YKL056C	61.2	88.4	0.831
18,978	5.95	135	YER067W	3.7	6.7	0.118
19,108	5.04	130	YLR109W	94.4	9.7	0.680
19,681	9.08	136	ATP7	11.0	NA[b,c]	0.246
20,505	6.07	111	GUK1	16.5	3.7	0.422
21,444	5.25	148	SAR1	5.4	10.4	0.455
21,583	4.98	95	TSA1	110.6	40.1	0.845
22,602	4.30	80	EFB1	66.1	23.8	0.875
23,079	6.29	112	SOD2	12.6	2.2	0.351
23,743	5.44	137	HSP26	NA[d]	0.7	0.434
24,033	5.97	96	ADK1	17.4	16.4	0.656
24,058	4.43	143	YKL117W	29.2	10.4	0.339
24,353	6.30	140	TFS1	8.1	0.7	0.146
24,662	5.85	99	URA5	25.4	6.0	0.359
24,808	6.33	97	GSP1	26.3	5.2	0.735
24,908	8.73	122	RPS5	18.6	NA[c]	0.899
25,081	4.65	81	MRP8	9.3	NA[c]	0.241
25,960	6.06	116	RPE1	5.8	0.7	0.372
26,378	9.55	127	RPS3	96.8	NA[c]	0.863
26,467	5.18	100	VMA4	10.5	3.7	0.427
26,661	5.84	98	TPI1	NA[d]	NA[c]	0.900
27,156	5.56	93	PRE8	6.9	0.7	0.129
27,334	6.13	115	YHR049W	18.4	2.2	0.520
27,472	5.33	92	YNL010W	31.6	3.7	0.421
27,480	8.95	123	GPM1	10.0	169.4	0.902
27,480	8.95	124	GPM1	231.4	169.4	0.902
27,480	8.95	125	GPM1	7.5	169.4	0.902
27,809	5.97	139	HOR2	5.7	0.7	0.381
27,874	4.46	78	YST1	13.6	52.8	0.805
28,595	4.51	41	PUP2	4.4	0.7	0.147
29,156	6.59	114	YMR226C	14.5	2.2	0.283
29,244	8.40	120	DPM1	5.0	11.2	0.362
29,443	5.91	48	PRE4	3.4	3.7	0.162
30,012	6.39	138	PRB1	21.2	1.5	0.449
30,073	4.63	77	BMH1	14.7	28.2	0.454
30,296	7.94	121	OMP2	67.4	41.6	0.499
30,435	6.34	89	GPP1	70.2	11.2	0.703
31,332	5.57	88	ILV6	13.9	3.0	0.402
32,159	5.46	113	IPP1	63.1	3.7	0.752
32,263	6.00	149	HIS1	22.4	4.5	0.232
33,311	5.35	84	SPE3	15.1	6.7	0.468
34,465	5.60	129	ADE1	8.7	5.2	0.305
34,762	5.32	85	SEC14	10.9	6.0	0.373
34,797	5.85	42	URA1	49.5	8.9	0.237
34,799	6.04	90	BEL1	103.2	81.0	0.875
35,556	5.97	43	YDL124W	6.4	4.5	0.206
35,619	8.41	59	TDH1	69.8	32.7[e]	0.940
35,650	5.49	68	CAR1	5.2	3.0	0.339
35,712	6.72	117	TDH2	49.6	473.0[e]	0.982
35,712	6.72	154	TDH2	863.5	473.0[e]	0.982
35,712	6.72	155	TDH2	79.4	473.0[e]	0.982
36,272	4.85	128	APA1	8.7	0.7	0.425
36,358	5.05	75	YJR105W	17.6	17.1	0.522
36,358	5.05	76	YJR105W	27.5	17.1	0.522
36,596	6.37	79	ADH2	58.9	260.0[e]	0.711
36,714	6.30	102	ADH1	746.1	260.0	0.913
36,714	6.30	103	ADH1	17.6	260.0	0.913
36,714	6.30	104	ADH1	61.4	260.0	0.913
36,714	6.30	105	ADH1	52.7	260.0	0.913
37,033	6.23	44	TAL1	44.8	3.7	0.701
37,796	7.36	57	IDH2	29.4	6.7	0.330
37,886	6.49	106	ILV5	76.0	4.5	0.892
38,700	7.83	55	BAT1	30.9	11.2	0.469
38,702	6.24	46	QCR2	NA[d]	2.2	0.326
39,477	5.58	86	FBA1	17.8	183.6	0.935
39,477	5.58	87	FBA1	427.2	183.6	0.935
39,540	6.50	150	HOM2	60.3	4.5	0.592
39,561	6.12	156	PSA1	96.4	27.5	0.718
41,158	6.01	49	YNL134C	14.9	1.5	0.316
41,623	7.18	58	BAT2	19.0	8.9	0.250
41,728	7.29	110	ERG10	24.1	4.5	0.543
41,900	5.42	74	TOM40	22.3	2.2	0.375
42,402	6.29	45	CYS3	6.7	8.9	0.621
42,883	5.63	67	DYS1	15.8	5.2	0.526
43,409	6.31	107	SER1	10.5	1.5	0.292
43,421	5.59	91	ERG6	2.2	14.1	0.408
44,174	7.32	56	YBR025C	13.1	6.0	0.684
44,682	4.99	72	TIF1	2.9	39.4	0.834
44,707	7.77	108	PGK1	23.7	165.7	0.897
44,707	7.77	109	PGK1	315.2	165.7	0.897
46,080	6.72	30	CAR2	15.4	NA[c]	0.495
46,383	8.52	53	IDP1	7.7	0.7	0.436
46,553	5.98	47	IDP2	32.4	NA[c]	0.197
46,679	6.39	50	ENO1	35.4	0.7	0.930
46,679	6.39	51	ENO1	6.6	0.7	0.930
46,679	6.39	52	ENO1	2.2	0.7	0.930
46,773	5.82	63	ENO2	15.5	289.1	0.960
46,773	5.82	64	ENO2	635.5	289.1	0.960
46,773	5.82	65	ENO2	93.0	289.1	0.960
46,773	5.82	66	ENO2	31.0	289.1	0.960
47,402	6.09	126	COR1	2.5	0.7	0.422
47,666	8.98	54	AAT2	11.7	6.0	0.338
48,364	5.25	73	WTM1	74.5	13.4	0.365
48,530	6.20	61	MET17	38.1	29.0	0.576
48,904	5.18	69	LYS9	16.2	3.7	0.463
48,987	4.90	153	SUP45	29.6	11.9	0.377
49,727	5.47	70	PRO2	13.6	5.2	0.297
49,912	9.27	62	TEF2	558.5	282.0	0.932
50,444	5.67	35	YDR190C	4.8	2.2	0.228
50,837	6.11	32	YEL047C	3.8	1.5	0.387
50,891	4.59	151	TUB2	11.2	7.4	0.404
51,547	6.80	27	LPD1	18.9	2.2	0.351
52,216	7.25	29	SHM2	19.7	7.4	0.722
52,859	5.54	37	YFR044C	30.2	6.7	0.442
53,798	5.19	71	HXK2	26.5	7.4	0.756
53,803	6.05	145	GYP6	4.4	0.7	0.147
54,403	5.29	39	ALD6	37.7	2.2	0.664
54,403	5.29	40	ALD6	6.6	2.2	0.664
54,502	6.20	31	ADE13	6.3	1.5	0.417
54,543	7.75	25	PYK1	225.3	101.8	0.965
54,543	7.75	26	PYK1	39.8	101.8	0.965
55,221	6.66	146	YEL071W	16.3	3.0	0.244
55,295	4.35	134	PDI1	66.2	14.1	0.589
55,364	5.98	24	GLK1	22.6	6.0	0.237
55,481	7.97	118	ATP1	21.6	2.2	0.637
55,886	6.47	28	CYS4	22.2	NA[c]	0.444
56,167	5.83	33	ARO8	14.3	3.0	0.324
56,167	5.83	34	ARO8	9.1	3.0	0.324
56,584	6.36	20	CYB2	18.9	NA[c]	0.259
57,366	5.53	60	FRS2	2.3	0.7	0.451
57,383	5.98	144	ZWF1	5.6	0.7	0.215
57,464	5.49	36	THR4	21.4	3.7	0.508
57,512	5.50	7	SRV2	6.5	NA[c]	0.260
57,727	4.92	152	VMA2	33.7	8.9	0.546
58,573	6.47	17	ACH1	4.4	1.5	0.327
58,573	6.47	18	ACH1	5.4	1.5	0.327
61,353	5.87	21	PDC1	6.5	200.7	0.962
61,353	5.87	22	PDC1	303.2	200.7	0.962
61,353	5.87	23	PDC1	16.3	200.7	0.962
61,649	5.54	38	CCT8	2.2	1.5	0.271

Continued

TABLE 1—Continued

Mol wt	pI	Spot no.	YPD gene name[a]	Protein abundance (10³ copies/cell)	mRNA abundance (copies/cell)	Codon bias
61,902	6.21	101	PDC5	4.3	NA[c]	0.828
62,266	6.19	16	ICL1	20.1	NA[c]	0.327
62,862	8.02	19	ILV3	5.3	4.5	0.548
63,082	6.40	119	PGM2	2.2	3.0	0.402
64,335	5.77	5	PAB1	30.4	1.5	0.616
66,120	5.42	8	STI1	6.7	0.7	0.313
66,120	5.42	9	STI1	6.4	0.7	0.313
66,450	5.29	141	SSB2	7.0	NA[c]	0.880
66,450	5.29	142	SSB2	2.3	NA[c]	0.880
66,456	5.23	10	SSB1	64.5	79.5	0.907
66,456	5.23	11	SSB1	59.0	79.5	0.907
66,456	5.23	12	SSB1	13.7	79.5	0.907
68,397	5.82	82	LEU4	3.1	3.0	0.407
69,313	4.90	13	SSA2	24.3	18.6	0.892
69,313	4.90	14	SSA2	77.1	18.6	0.892
74,378	8.46	15	YKL029C	2.8	3.7	0.353
75,396	5.82	6	GRS1	5.5	7.4	0.500
85,720	6.25	1	MET6	2.0	NA[c]	0.772
85,720	6.25	2	MET6	10.9	NA[c]	0.772
85,720	6.25	3	MET6	1.4	NA[c]	0.772
93,276	6.11	131	EFT1	17.9	41.6	0.890
93,276	6.11	132	EFT1	5.7	41.6	0.890
102,064[e]	6.61[e]	94	ADE3	4.8	5.2	0.423
107,482[e]	5.33[e]	4	MCM3	2.7	NA[c]	0.240

[a] YPD gene names are available from the YPD website (39).

[b] NA, calculation could not be performed or was not available.

[c] mRNA data inconclusive or NA.

[d] No methionines in predicted ORF; therefore, protein concentration was not determined.

[e] Measured molecular weight or pI did not match theoretical molecular weight or pI.

Protein quantitation. [³⁵S]methionine-labeled gels were exposed to X-ray film overnight, and then the silver stain and film were used to excise 156 spots of varying intensities, molecular weights, and pIs. The excised spots were placed in 0.6-ml microcentrifuge tubes, and scintillation cocktail (100 μl) was added. The samples were vortexed and counted. In addition, two parallel gels were electroblotted to polyvinylidene difluoride membranes. The membranes were exposed to X-ray film, and four intense single spots were excised from each membrane and subjected to amino acid analysis. For these four spots, a mean of 209 ± 4 cpm/pmol of protein/methionine was found. This number was used to quantitate all remaining spots in conjunction with the number of methionines present in the protein.

To ensure that proteins were labeled to equilibrium, parallel 2D gels were prepared and run on yeast metabolically labeled for 1, 2, 6, or 18 h. The corresponding 156 spots were excised from each gel, and radioactivity was measured by liquid scintillation counting for each spot. Calculated protein levels were highly reproducible for all time points measured after 1 h.

Calculation of codon bias and predicted half-life. Codon bias values were extracted from the YPD spreadsheet (17). Protein half-lives were calculated based on the N-end rule (33). When the N-terminal processing was not known experimentally, it was predicted based on the affinity of methionine aminopeptidase (31).

RESULTS

Characteristics of proteome approach. Nearly every facet of proteome analysis hinges on the unambiguous identification of large numbers of expressed proteins in cells. Several techniques have been described previously for the identification of proteins separated by 2DE, including N-terminal and internal sequencing (1, 2), amino acid analysis (38), and more recently mass spectrometry (25). We utilized techniques based on mass spectrometry because they afford the highest levels of sensitivity and provide unambiguous identification. The specific procedure used is schematically illustrated in Fig. 1 and is based on three principles. First, proteins are removed from the gel by

proteolytic in-gel digestion, and the resulting peptides are separated by on-line capillary high-performance liquid chromatography. Second, the eluting peptides are ionized and detected, and the specific peptide ions are selected and fragmented by the mass spectrometer. To achieve this, the mass spectrometer switches between the MS mode (for peptide mass identification) and the MS/MS mode (for peptide characterization and sequencing). Selected peptides are fragmented by a process called collision-induced dissociation (CID) to generate a tandem mass spectrum (MS/MS spectrum) that contains the peptide sequence information. Third, individual CID mass spectra are then compared by computer algorithms to predicted spectra from a sequence database. This results in the identification of the peptide and, by association, the protein(s) in the spot. Unambiguous protein identification is attained in a single analysis by the detection of multiple peptides derived from the same protein.

Protein identification. Yeast total cell protein lysate (40 μg), metabolically labeled with [³⁵S]methionine, was electrophoretically separated by isoelectric focusing in the first dimension and by SDS–10% polyacrylamide gel electrophoresis in the second dimension. Proteins were visualized by silver staining and by autoradiography. Of the more than 1,000 proteins visible by silver staining, 156 spots were excised from the gel and subjected to in-gel tryptic digestion, and the resulting peptides were analyzed and identified by microspray LC-MS/MS techniques as described above. The proteins in this study were all identified automatically by computer software with no human interpretation of mass spectra. They are indicated in Fig. 2 and detailed in Table 1.

The CID spectra shown in Fig. 3 indicate that the quality of the identification data generated was suitable for unambiguous protein identification. The spectra represent the amino acid sequences of tryptic peptides NSGDIVNLGSIAGR (Fig. 3A) and FAVGAFTDSLR (Fig. 3B). Both peptides were derived from protein S57593 (hypothetical protein YMR226C), which migrated to spot 114 (molecular weight, 29,156; pI, 6.59) in the 2D gel in Fig. 2. Five other peptides from the same analysis were also computer matched to the same protein sequence.

Protein and mRNA quantitation. For the 156 genes investigated, the protein expression levels ranged from 2,200 (PGM2) to 863,000 (TDH2/TDH3) copies/cell. The levels of mRNA for each of the genes identified were calculated from SAGE frequency tables (35). These tables contain the mRNA levels for 4,665 genes in yeast strain YPH499 grown to mid-log phase in YPD medium on glucose as a carbon source. In some instances, the mRNA levels could not be calculated for reasons stated in Materials and Methods. For the proteins analyzed in this study, mean transcript levels varied from 0.7 to 473 copies/cell.

Selection of the sample population for mRNA-protein expression level correlation. The protein spots selected for identification were selected from spots visible by silver staining in the 2D gel. An attempt was made not to include spots where overlap with other spots was readily apparent. The number of proteins identified was 156 (Table 1). Some proteins migrated to more than one spot (presumably due to differential protein processing or modifications), and protein levels from these spots were calculated by integrating the intensities of the different spots. The 156 protein spots analyzed represented the products of 128 different genes. Genes were excluded from the correlation analysis only if part of the data set was missing; i.e., genes were excluded if (i) no mRNA expression data were available for the protein or putative SAGE tags were ambiguous, (ii) the amino acid sequence did not contain methionine, (iii) more than a single protein was conclusively identified as
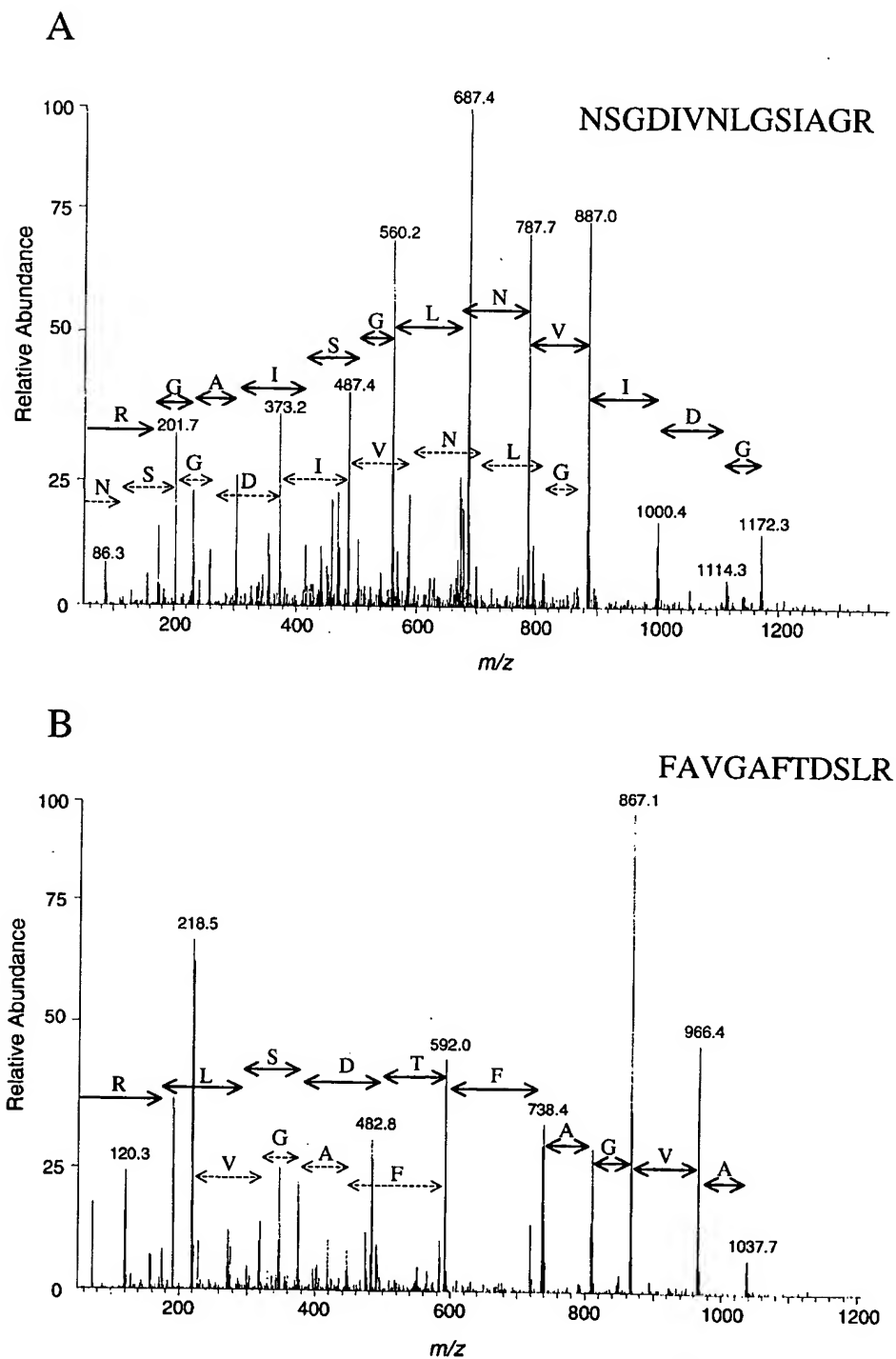
A

B

FIG. 3. Tandem mass (MS/MS) spectra resulting from analysis of a single spot on a 2D gel. The first quadrupole selected a single mass-to-charge ratio (m/z) of 687.2 (A) or 592.6 (B), while the collision cell was filled with argon gas, and a voltage which caused the peptide to undergo fragmentation by CID was applied. The third quadrupole scanned the mass range from 50 to 1,400 m/z. The computer program Sequest (8) was utilized to match MS/MS spectra to amino acid sequence by database searching. Both spectra matched peptides from the same protein, S57593 (yeast hypothetical protein YMR226C). Five other peptides from the same analysis were matched to the same protein.

migrating to the same gel spot, or (iv) the theoretical and observed pIs and molecular weights could not be reconciled. After these criteria were applied, the number of genes used in the correlation analysis was 106.

Codon bias and predicted half-lives. Codon bias is thought to be an indicator of protein expression, with highly expressed proteins having large codon bias values. The codon bias distribution for the entire set of more than 6,000 predicted yeast

gene ORFs is presented in Fig. 4A. The interval with the largest frequency of genes is between the codon bias values of 0.0 and 0.1. This segment contains more than 2,500 genes. The distribution of the codon bias values of the 128 different genes found in this study (all protein spots from Fig. 2) is shown in Fig. 4B, and protein half-lives (predicted from applying the N-end rule [33] to the experimentally determined or predicted protein N termini) are shown in Fig. 4C. No genes were identified with codon bias values less than 0.1 even though thousands of genes exist in this category. In addition, nearly all of the proteins identified had long predicted half-lives (greater than 30 h).

Correlation of mRNA and protein expression levels. The correlation between mRNA and protein levels of the genes selected as described above is shown in Fig. 5. For the entire group (106 genes) for which a complete data set was generated, there was a general trend of increased protein levels resulting from increased mRNA levels. The Pearson product moment correlation coefficient for the whole data set (106 genes) was 0.935. This number is highly biased by a small number of genes with very large protein and message levels. A more representative subset of the data is shown in the inset of Fig. 5. It shows genes for which the message level was below 10 copies/cell and includes 69% (73 of 106 genes) of the data used in the study. The Pearson product moment correlation coefficient for this data set was only 0.356. We also found that levels of protein expression coded for by mRNA with comparable abundance varied by as much as 30-fold and that the mRNA levels coding for proteins with comparable expression levels varied by as much as 20-fold.

The distortion of the correlation value induced by the uneven distribution of the data points along the x axis is further demonstrated by the analysis in Fig. 6. The 106 samples included in the study were ranked by protein abundance, and the Pearson product moment correlation coefficient was repeatedly calculated after including progressively more, and higher-abundance, proteins in each calculation. The correlation values remained relatively stable in the range of 0.1 to 0.4 if the lowest-expressed 40 to 95 proteins used in this study were included. However, the correlation value steadily climbed by the inclusion of each of the 11 very highly expressed proteins.

Correlation of protein and mRNA expression levels with codon bias. Codon bias is the propensity for a gene to utilize the same codon to encode an amino acid even though other codons would insert the identical amino acid in the growing polypeptide sequence. It is further thought that highly expressed proteins have large codon biases (3). To assess the value of codon bias for predicting mRNA and protein levels in exponentially growing yeast cells, we plotted the two experimental sets of data versus the codon bias (Fig. 7). The distribution patterns for both mRNA and protein levels with respect to codon bias were highly similar. There was high variability in the data within the codon bias range of 0.8 to 1.0. Although a large codon bias generally resulted in higher protein and message expression levels, codon bias did not appear to be predictive of either protein levels or mRNA levels in the cell.

DISCUSSION

The desired end point for the description of a biological system is not the analysis of mRNA transcript levels alone but also the accurate measurement of protein expression levels and their respective activities. Quantitative analysis of global mRNA levels currently is a preferred method for the analysis of the state of cells and tissues (11). Several methods which either provide absolute mRNA abundance (34, 35) or relative
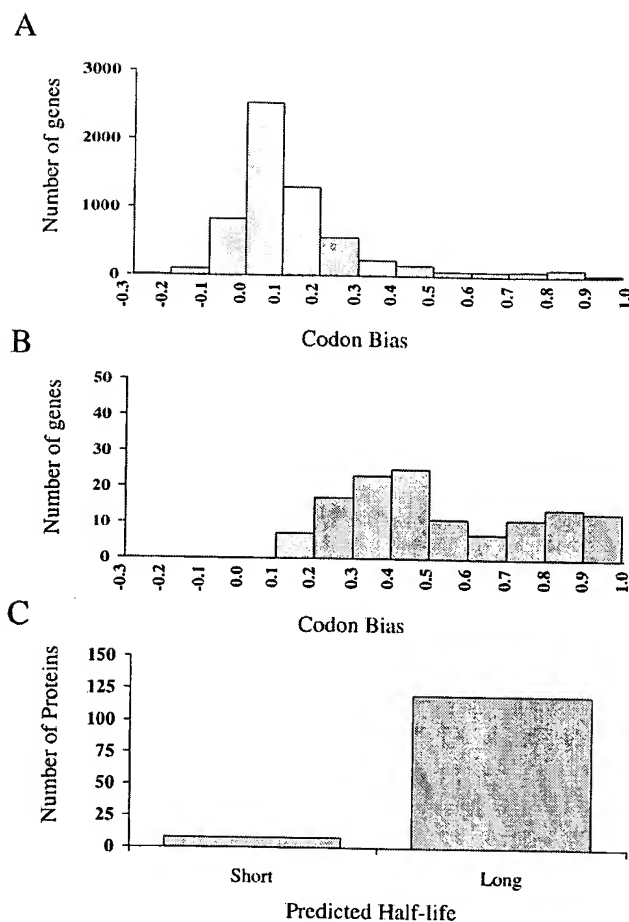
FIG. 4. Current proteome analysis technology utilizing 2DE without preenrichment samples mainly highly expressed and long-lived proteins. Genes encoding highly expressed proteins generally have large codon bias values. (A) Distribution of the yeast genome (more than 6,000 genes) based on codon bias. The interval with the largest frequency of genes is 0.0 to 0.1, with more than 2,500 genes. (B) Distribution of the genes from identified proteins in this study based on codon bias. No genes with codon bias values less than 0.1 were detected in this study. (C) Distribution of identified proteins in this study based on predicted half-life (estimated by N-end rule).

mRNA levels in comparative analyses (20, 27) have been described elsewhere. The techniques are fast and exquisitely sensitive and can provide mRNA abundance for potentially any expressed gene. Measured mRNA levels are often implicitly or explicitly extrapolated to indicate the levels of activity of the corresponding protein in the cell. Quantitative analysis of protein expression levels (proteome analysis) is much more time-consuming because proteins are analyzed sequentially one by one and is not general because analyses are limited to the relatively highly expressed proteins. Proteome analysis does, however, provide types of data that are of critical importance for the description of the state of a biological system and that are not readily apparent from the sequence and the level of expression of the mRNA transcript. This study attempts to examine the relationship between mRNA and protein expression levels for a large number of expressed genes in cells representing the same state.

Limits in the sensitivity of current protein analysis technology precluded a completely random sampling of yeast proteins. We therefore based the study on those proteins visible by silver
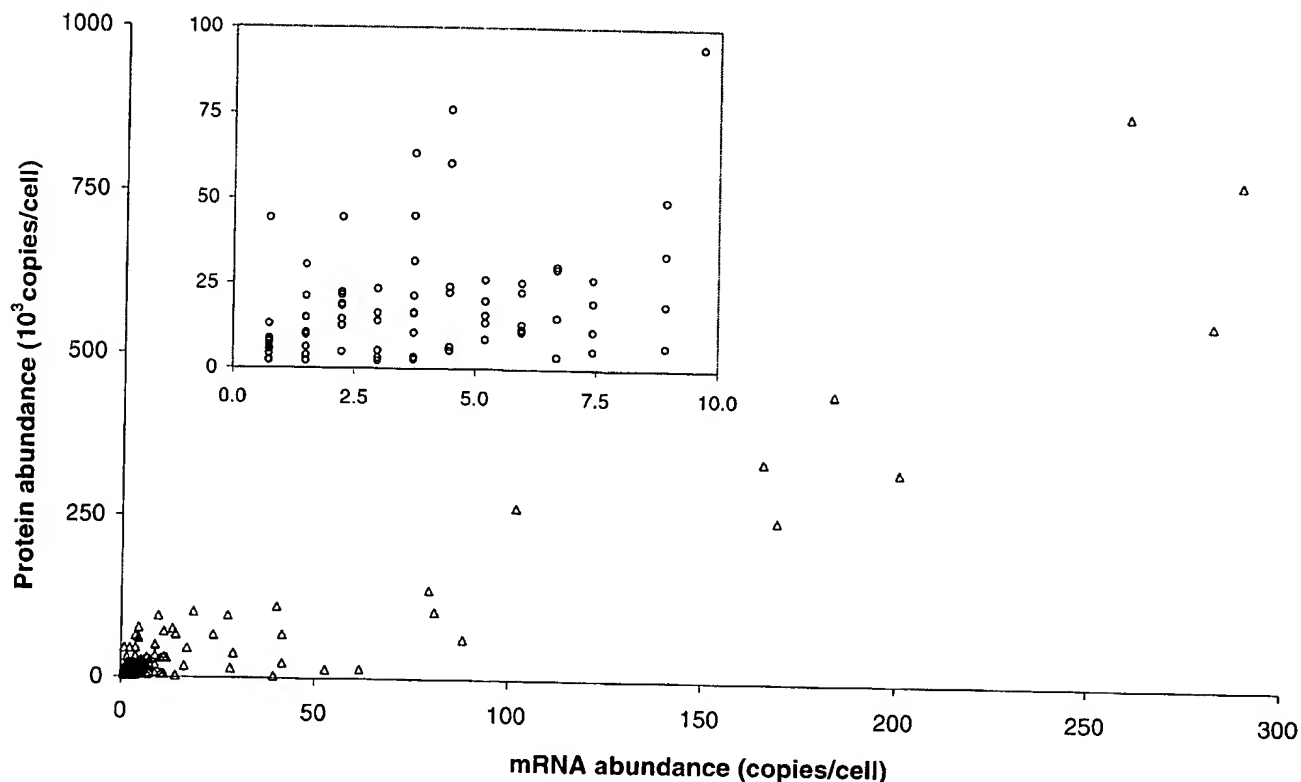
FIG. 5. Correlation between protein and mRNA levels for 106 genes in yeast growing at log phase with glucose as a carbon source. mRNA and protein levels were calculated as described in Materials and Methods. The data represent a population of genes with protein expression levels visible by silver staining on a 2D gel chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities. The inset shows the low-end portion of the main figure. It contains 69% of the original data set. The Pearson product moment correlation for the entire data set was 0.935. The correlation for the inset containing 73 proteins (69%) was only 0.356.

staining on a 2D gel. Of the more than 1,000 visible spots, 156 were chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities displayed on the 2D protein pattern. The genes identified in this study shared a number of properties. First, all of the proteins in this study had a codon bias of greater than 0.1 and 93% were greater than 0.2 (Fig. 4B). Second, with few exceptions, the proteins in this study had long predicted half-lives according to the N-end rule (Fig. 4C). Third, low-abundance proteins with regulatory functions such as transcription factors or protein kinases were not identified.

Because the population of proteins used in this study appears to be fairly homogeneous with respect to predicted half-life and codon bias, it might be expected that the correlation of the mRNA and protein expression levels would be stronger for this population than for a random sample of yeast proteins. We tested this assumption by evaluating the correlation value if different subsets of the available data were included in the calculation. The 106 proteins were ranked from lowest to highest protein expression level, and the trend in the correlation value was evaluated by progressively including more of the higher-abundance proteins in the calculation (Fig. 6). The correlation value when only the lower-abundance 40 to 93 proteins were examined was consistently between 0.1 and 0.4. If the 11 most abundant proteins were included, the correlation steadily increased to 0.94. We therefore expect that the correlation for all yeast proteins or for a random selection would be less than 0.4. The observed level of correlation between mRNA and protein expression levels suggests the importance

of posttranslational mechanisms controlling gene expression. Such mechanisms include translational control (15) and control of protein half-life (33). Since these mechanisms are also active in higher eukaryotic cells, we speculate that there is no predictive correlation between steady-state levels of mRNA and those of protein in mammalian cells.

Like other large-scale analyses, the present study has several potential sources of error related to the methods used to determine mRNA and protein expression levels. The mRNA levels were calculated from frequency tables of SAGE data. This method is highly quantitative because it is based on actual sequencing of unique tags from each gene, and the number of times that a tag is represented is proportional to the number of mRNA molecules for a specific gene. This method has some limitations including the following: (i) the magnitude of the error in the measurement of mRNA levels is inversely proportional to the mRNA levels, (ii) SAGE tags from highly similar genes may not be distinguished and therefore are summed, (iii) some SAGE tags are from sequences in the 3' untranslated region of the transcript, (iv) incomplete cleavage at the SAGE tag site by the restriction enzyme can result in two tags representing one mRNA, and (v) some transcripts actually do not generate a SAGE tag (34, 35).

For the SAGE method, the error associated with a value increases with a decreasing number of transcripts per cell. The conclusions drawn from this study are dependent on the quality of the mRNA levels from previously published data (35). Since more than 65% of the mRNA levels included in this study were calculated to 10 copies/cell or less (40% were less
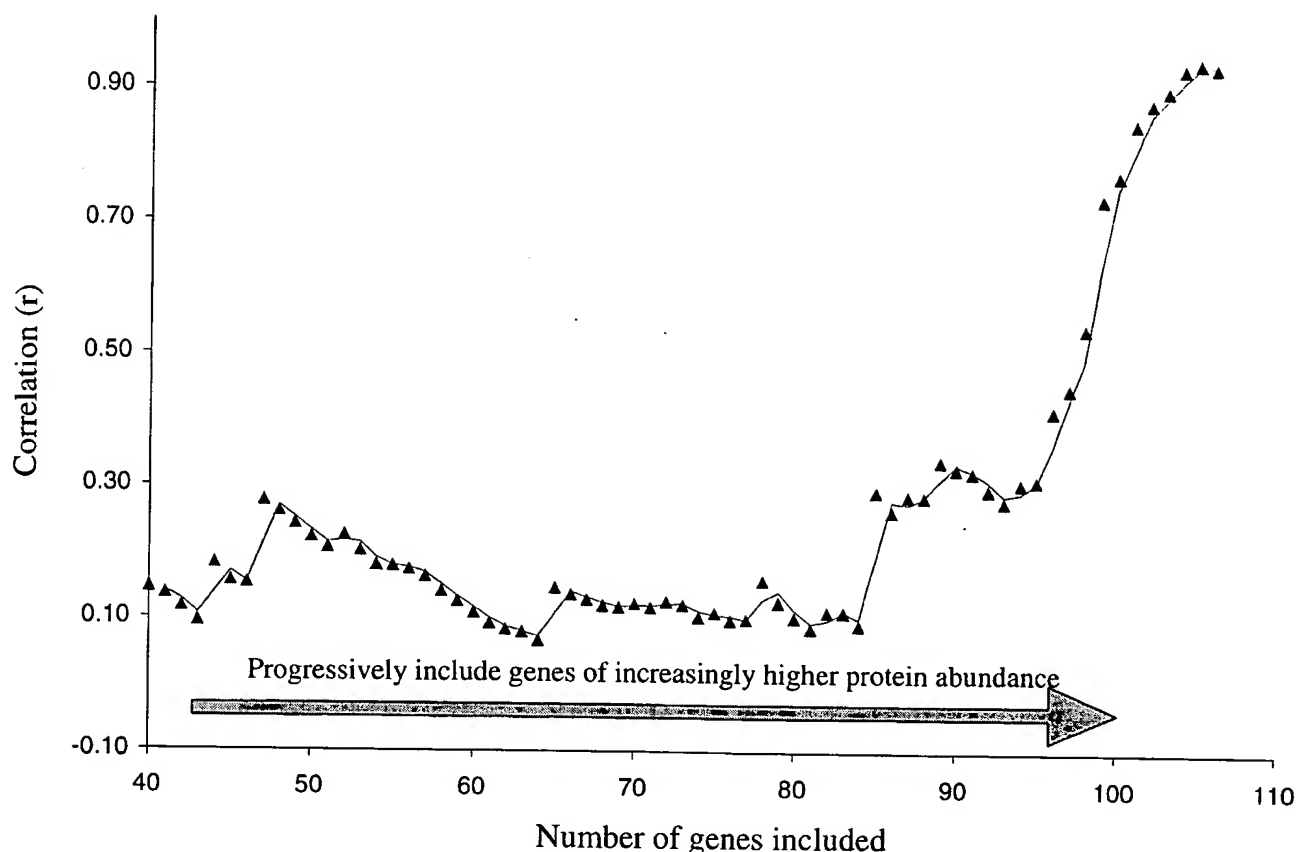
FIG. 6. Effect of highly abundant proteins on Pearson product moment correlation coefficient for mRNA and protein abundance in yeast. The set of 106 genes was ranked according to protein abundance, and the correlation value was calculated by including the 40 lowest-abundance genes and then progressively including the remaining 66 genes in order of abundance. The correlation value climbs as the final 11 highly abundant proteins are included.

than 4 copies/cell), the error associated with these values may be quite large. The mRNA levels were calculated from more than 20,000 transcripts. Assuming that the estimate of 15,000 mRNA molecules per cell is correct (16), this would mean that mRNA transcripts present at only a single copy per cell would be detected 72% of the time (35). The mRNA levels for each gene were carefully scrutinized, and only mRNA levels for which a high degree of confidence existed were included in the correlation value.

Protein abundance was determined by metabolic radiolabeling with [^{35}S]methionine. The calculation required knowledge of three variables: the number of methionines in the mature protein, the radioactivity contained in the protein, and the specific activity of the radiolabel normalized per methionine. The number of methionines per protein was determined from the amino acid sequence of the proteins identified by tandem mass spectrometry. For some proteins, it was not known whether the methionine of the nascent polypeptide was processed away. The N termini of those proteins were predicted based on the specificity of methionine aminopeptidase (31). If the N-terminal processing did not conform to the predicted specificity of processing enzymes, the calculation of the number of methionines would be affected. This discrepancy would affect most the quantitation of a protein with a very low number of methionines. The average number of calculated methionines per protein in this study was 7.2. We therefore expect the potential for erroneous protein quantitation due to unusual N-terminal processing to be small.

The amount of radioactivity contained in a single spot might be the sum of the radioactivity of comigrating proteins. Because protein identification was based on tandem mass spectrometric techniques, comigrating proteins could be identified. However, comigrating proteins were rarely detected in this study, most likely because relatively small amounts of total protein (40 μg) were initially loaded onto the gels, which resulted in highly focused spots containing generally 1 to 25 ng of protein. Because of the relatively small amount loaded, the concentrations of any potentially comigrating protein would likely be below the limit of detection of the mass spectrometry technique used in this study (1 to 5 ng) and below the limit of visualization by silver staining (1 to 5 ng). In the overwhelming majority of the samples analyzed, numerous peptides from a single protein were detected. It is assumed that any comigrating proteins were at levels too low to be detected and that their influence in the calculation would be small.

The specific activity of the radiolabel was determined by relating the precise amount of protein present in selected spots of a parallel gel, as determined by quantitative amino acid composition analysis, to the number of methionines present in the sequence of those proteins and the radioactivity determined by liquid scintillation counting. It is possible that the resulting number might be influenced by unavoidable losses inherent in the amino acid analysis procedure applied. Because four different proteins were utilized in the calculation and the experiment was done in duplicate, the specific activity calculated is thought to be highly accurate. Indeed, the specific
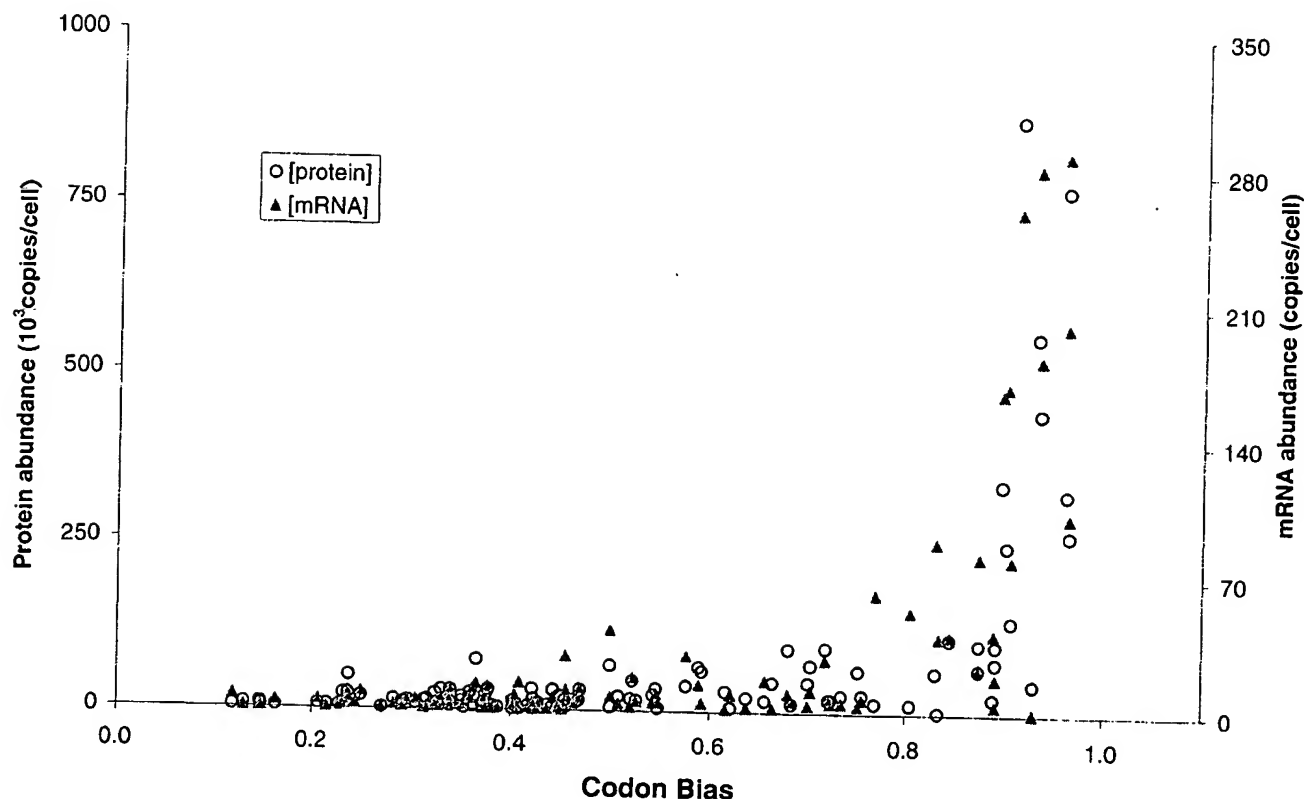
FIG. 7. Relationship between codon bias and protein and mRNA levels in this study. Yeast mRNA and protein expression levels were calculated as described in Materials and Methods. The data represent the same 106 genes as in Fig. 5.

activities calculated for each of the four proteins varied by less than 10%. Any inconsistencies in the calculation of the specific activity would result in differences in the absolute levels calculated but not in the relative numbers and would therefore not influence the correlation value determined.

The protein quantitative method used eliminates a number of potential errors inherent in previous methods for the quantitation of proteins separated by 2DE, such as preferential protein staining and bias caused by inequalities in the number of radiolabeled residues per protein. Any 2D gel-based method of quantitation is complicated by the fact that in some cases the translation products of the same mRNA migrated to different spots. One major reason is posttranslational modification or processing of the protein. Also, artifactual proteolysis during cell lysis and sample preparation can lead to multiple resolved forms of the protein. In such cases, the protein levels of spots coded for by the same mRNA were pooled. In addition, the existence of other spots coded for by the same mRNA that were not analyzed by mass spectrometry or that were below the limit of detection for silver staining cannot be ruled out. However, since this study is based on a class of highly expressed proteins, the presence of undetected minor spots below silver staining sensitivity corresponding to a protein analyzed in the study would generally cause a relatively small error in protein quantitation.

Codon bias is a measure of the propensity of an organism to selectively utilize certain codons which result in the incorporation of the same amino acid residue in a growing polypeptide chain. There are 61 possible codons that code for 20 amino acids. The larger the codon bias value, the smaller the number of codons that are used to encode the protein (19). It is

thought that codon bias is a measure of protein abundance because highly expressed proteins generally have large codon bias values (3, 13).

Nearly all of the most highly expressed proteins had codon bias values of greater than 0.8. However, we detected a number of genes with high codon bias and relative low protein abundance (Fig. 7). For example, the expressed gene with both the second largest protein and mRNA levels in the study was ENO2_YEAST (775,000 and 289.1 copies/cell, respectively). ENO1_YEAST was also present in the gel at much lower protein and mRNA levels (44,200 and 0.7 copies/cell, respectively). The codon bias values for ENO2 and ENO1 are similar (0.96 and 0.93, respectively), but the expression of the two genes is differentially regulated. Specifically, ENO1_YEAST is glucose repressed (6) and was therefore present in low abundance under the conditions used. Other genes with large codon bias values that were not of high protein abundance in the gel include EFT1, TIF1, HXK2, GSP1, EGD2, SHM2, and TAL1. We conclude that merely determining the codon bias of a gene is not sufficient to predict its protein expression level.

Interestingly, codon bias appears to be an excellent indicator of the boundaries of current 2D gel proteome analysis technology. There are thousands of genes with expressed mRNA and likely expressed protein with codon bias values less than 0.1 (Fig. 4A). In this study, we detected none of them, and only a very small percentage of the genes detected in this study had codon bias values between 0.1 and 0.2 (Fig. 4B). Indeed, in every examined yeast proteome study (5, 7, 13, 28) where the combined total number of identified proteins is 300 to 400, this same observation is true. It is expected that for the more complex cells of higher eukaryotic organisms the detection of

low-abundance proteins would be even more challenging than for yeast. This indicates that highly abundant, long-lived proteins are overwhelmingly detected in proteome studies. If proteome analysis is to provide truly meaningful information about cellular processes, it must be able to penetrate to the level of regulatory proteins, including transcription factors and protein kinases. A promising approach is the use of narrow-range focusing gels with immobilized pH gradients (IPG) (23). This would allow for the loading of significantly more protein per pH unit covered and also provide increased resolution of proteins with similar electrophoretic mobilities. A standard pH gradient in an isoelectric focusing gel covers a 7-pH-unit range (pH 3 to 10) over 18 cm. A narrow-range focusing gel might expand the range to 0.5 pH units over 18 cm or more. This could potentially increase by more than 10-fold the number of proteins that can be detected. Clearly, current proteome technology is incapable of analyzing low-abundance regulatory proteins without employing an enrichment method for relatively low-abundance proteins. In conclusion, this study examined the relationship between yeast protein and message levels and revealed that transcript levels provide little predictive value with respect to the extent of protein expression.

ACKNOWLEDGMENTS

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Exhibit 13

TECHNICAL UPDATE

FROM YOUR LABORATORY SERVICES PROVIDER

HER-2/neu Breast Cancer Predictive Testing

Julie Sanford Hanna, Ph.D. and Dan Mornin, M.D.

EACH YEAR, OVER 182,000 WOMEN in the United States are diagnosed with breast cancer, and approximately 45,000 die of the disease.[1] Incidence appears to be increasing in the United States at a rate of roughly 2% per year. The reasons for the increase are unclear, but non-genetic risk factors appear to play a large role.[2]

Five-year survival rates range from approximately 65%-85%, depending on demographic group, with a significant percentage of women experiencing recurrence of their cancer within 10 years of diagnosis. One of the factors most predictive for recurrence once a diagnosis of breast cancer has been made is the number of axillary lymph nodes to which tumor has metastasized. Most node-positive women are given adjuvant therapy, which increases their survival. However, 20%-30% of patients without axillary node involvement also develop recurrent disease, and the difficulty lies in how to identify this high-risk subset of patients, These patients could benefit from increased surveillance, early intervention, and treatment.

Prognostic markers currently used in breast cancer recurrence prediction include tumor size, histological grade, steroid hormone receptor status, DNA ploidy, proliferative index, and cathepsin D status. Expression of growth factor receptors and over-expression of the HER-2/neu oncogene have also been identified as having value regarding treatment regimen and prognosis.

HER-2/neu (also known as c-erbB2) is an oncogene that encodes a transmembrane glycoprotein that is homologous to, but distinct from, the epidermal growth factor receptor. Numerous studies have indicated that high levels of expression of this protein are associated with rapid tumor growth, certain forms of therapy resistance, and shorter disease-free survival. The gene has been shown to be amplified and/or overexpressed in 10%-30% of invasive breast cancers and in 40%-60% of intraductal breast carcinoma.[3]

There are two distinct FDA-approved methods by which HER-2/neu status can be evaluated: immunohistochemistry (IHC, HercepTest™) and FISH (fluorescent in situ hybridization, PathVysion™ Kit). Both methods can be performed on archived and current specimens. The first method allows visual assessment of the amount of HER-2/neu protein present on the cell membrane. The latter method allows direct quantification of the level of gene amplification present in the tumor, enabling differentiation between low- versus high-amplification. At least one study has demonstrated a difference in recurrence risk in women younger than 40 years of age for low- versus high-amplified tumors (54.5% compared to 85.7%); this is compared to a recurrence rate of 16.7% for patients with no HER-2/neu gene amplification.[4] HER-2/neu status may be particularly important to establish in women with small (≤ 1 cm) tumor size.

The choice of methodology for determination of HER-2/neu status depends in part on the clinical setting. FDA approval for the Vysis FISH test was granted based on clinical trials involving 1549 node-positive patients. Patients received one of three different treatments consisting of different doses of cyclophosphamide, Adriamycin, and 5-fluorouracil (CAF). The study showed that patients with amplified HER-2/neu benefited from treatment with higher doses of adriamycin-based therapy, while those with normal HER-2/neu levels did not. The study therefore identified a sub-set of women, who because they did not benefit from more aggressive treatment, did not need to be exposed to the associated side effects. In addition, other evidence indicates that HER-2/neu amplification in node-negative patients can be used as an independent prognostic indicator for early recurrence, recurrent disease at any time and disease-related death.[5] Demonstration of HER-2/neu gene amplification by FISH has also been shown to be of value in predicting response to chemotherapy in stage-2 breast cancer patients.

Selection of patients for Herceptin© (Trastuzumab) monoclonal antibody therapy, however, is based upon demonstration of HER-2/neu protein overexpression using HercepTest™. Studies using Herceptin© in patients with metastatic breast cancer show an increase in time to disease progression, increased response rate to chemotherapeutic agents and a small increase in overall survival rate. The FISH assays have not yet been approved for this purpose, and studies looking at response to Herceptin© in patients with or without gene amplification status determined by FISH are in progress.

In general, FISH and IHC results correlate well. However, subsets of tumors are found which show discordant results; i.e., protein overexpression without gene amplification or lack of protein overexpression with gene amplification. The clinical significance of such results is unclear. Based on the above considerations, HER-2/neu testing at SHMC/PAML will utilize immunohistochemistry (HercepTest©) as a screen, followed by FISH in IHC-negative cases. Alternatively, either method may be ordered individually depending on the clinical setting or clinician preference.

CPT code information

HER-2/neu via IHC

88342   (including interpretive report)

HER-2/neu via FISH

88271×2   Molecular cytogenetics, DNA probe, each
88274   Molecular cytogenetics, interphase in situ hybridization, analyze 25-99 cells
88291   Cytogenetics and molecular cytogenetics, interpretation and report

Procedural Information

Immunohistochemistry is performed using the FDA-approved DAKO antibody kit, Herceptest©. The DAKO kit contains reagents required to complete a two-step immunohistochemical staining procedure for routinely processed, paraffin-embedded specimens. Following incubation with the primary rabbit antibody to human HER-2/neu protein, the kit employs a ready-to-use dextran-based visualization reagent. This reagent consists of both secondary goat anti-rabbit antibody molecules with horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugated antibody. Enzymatic conversion of the subsequently added chromogen results in formation of visible reaction product at the antigen site. The specimen is then counterstained; a pathologist using light-microscopy interprets results.

FISH analysis at SHMC/PAML is performed using the FDA-approved PathVysion™ HER-2/neu DNA probe kit, produced by Vysis, Inc. Formalin fixed, paraffin-embedded breast tissue is processed using routine histological methods, and then slides are treated to allow hybridization of DNA probes to the nuclei present in the tissue section. The Pathvysion™ kit contains two direct-labeled DNA probes, one specific for the alphoid repetitive DNA (CEP 17, spectrum orange) present at the chromosome-17 centromere and the second for the HER-2/neu oncogene located at 17q11.2-12 (spectrum green). Enumeration of the probes allows a ratio of the number of copies of chromosome 17 to the number of copies of HER-2/neu to be obtained; this enables quantification of low versus high amplification levels, and allows an estimate of the percentage of cells with HER-2/neu gene amplification. The clinically relevant distinction is whether the gene amplification is due to increased gene copy number on the two chromosome 17 homologues normally present or an increase in the number of chromosome 17s in the cells. In the majority of cases, ratio equivalents less than 2.0 are indicative of a normal/negative result, ratios of 2.1 and over indicate that amplification is present and to what degree. Interpretation of this data will be performed and reported from the Vysis-certified Cytogenetics laboratory at SHMC.

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